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Local and systemic inflammation in oesophageal disease

University of Dublin, Trinity College

M.D. Thesis

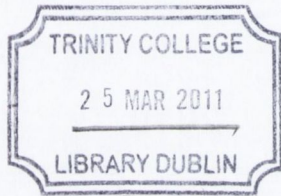## 2010

James Oliver Murphy

MB BCh BAO (Hons), MRCSI

Department of Surgery
Trinity Centre
St. James's Hospital
Dublin 8
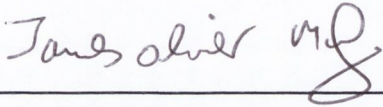
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Summary of thesis

Part A   Animal studies (Chapters 3 and 4)

Background: Reflux-induced injury and oxidative stress result in oesophageal inflammation and the potential for progression to intestinal metaplasia and adenocarcinoma. Proton-pump inhibitors represent the standard medical approach, but anti-inflammatories and antioxidants offer novel therapeutic possibilities.

Methods: A surgical oesophagojejunostomy model of reflux in the rat was established for the first time in Ireland. The dosage and administration method for an antioxidant and a COX-2 inhibitor were optimised for use in this reflux model. Six weeks after an oesophagojejunostomy reflux procedure, female Wistar rats (n=100) were randomized to either receive an antioxidant (vitamin C 8mg or 28mg/day), a COX-2 inhibitor (Rofecoxib 1mg/day) or no therapy. After sacrifice 16 weeks later, esophageal injury was scored using pathologic and image analysis scoring.

Results: A surgical oesophagojejunostomy model of reflux was successfully established and used to study the effects of a COX-2 inhibitor and an antioxidant on oesophageal injury. Oesophagitis was present in all 63 animals completing the study and was severe in 27 (43%). No animal developed metaplasia or tumour. The extent of inflammation and esophageal ulceration were not significantly different between experimental groups.

Conclusions: In this model of reflux injury, antioxidants and COX-2 inhibitors failed to ameliorate the severe inflammation induced. Further experimental designs should evaluate these novel approaches in less severe experimental models.

Part B   Human studies (Chapters 5 and 6)

Background: A multimodality approach is increasingly utilised in gastrointestinal cancer, however the impact of neoadjuvant chemoradiotherapy (CRT) on the postoperative response to surgery is unknown. The cytokine response following major surgery has been broadly studied; however this has mostly been performed using superseded technology to measure a limited number cytokines at protein level.

Methods: Serum and blood were collected from 31 non-randomised, stage-matched oesophageal cancer patients treated with surgery alone or a multimodal treatment. Blood was collected pre-operatively and on postoperative days 1 and 7 following oesophagectomy. "Biochip" array technology was used to measure the levels of 12 cytokines in serum samples; IL-2, IL-4, IL-6, IL-8, IL-10, VEGF, IFN-γ, TNF-α, IL-1α, IL-1β, MCP-1, EGF. Real time quantitative polymerase chain reaction (RQ PCR) was performed

for IL-10 and TNF-α mRNA expression in ribonucleic acid (RNA) extracted from whole blood and peripheral blood mononuclear cells (PBMCs), using ABL as an endogenous control. The cytokine responses following oesophagectomy were examined in surgery and multimodal patients and the groups were compared to detect differences between treatment strategies.

Results: There were significant changes detected in the levels of 6 serum cytokines following oesophagectomy in both patient groups: IL-6, IL-8, IL-10, VEGF, MCP1 and EGF. No differences between the surgery and multimodal groups were found for the serum results. When mRNA expression levels were compared between the surgery and multimodal groups, increased preoperative IL-10 and TNF-α expression levels were found in multimodal patients. However, overall IL-10 and TNF-α expression and the ratio of TNF-α: IL-10 expression were similar in both groups.

Conclusion: Significant cytokine responses were found in surgery and multimodal patients following oesophagectomy at protein and RNA levels. There were significantly increased preoperative TNF-α and IL-10 mRNA expression levels in multimodal patients relative to surgery patients, however overall TNF-α and IL-10 expression and the ratio of TNF-α: IL-10 expression was the same. Parallel protein and RNA detection using recently developed technologies has produced a new understanding of the complex cytokine following oesophagectomy. Differences found between surgery and multimodal patients in this study may reflect differences in complication rates and survival between these groups.

Publications to date from this thesis

Murphy JO, Ravi N, Byrne PJ, McDonald GSA, Reynolds JV. Neither antioxidants nor COX-2 inhibition protect against esophageal inflammation in an experimental model of severe reflux. J Surg Res 2007: 142(1): 20-27. PMID: 17543990.

Murphy JO, Miller N, Pidgeon GP, Ravi N, Reynolds JV. Serum and cellular RNA cytokine response following oesophagectomy in patients treated with a multimodal regimen compared with surgery. Submitted for publication.

Presentations from this thesis

Association of Surgeons of Great Britain and Ireland, March 2006

Parallel gene and protein expression analysis of the immune response to major surgery in oesophageal cancer patients treated with surgery alone or a multimodality regimen

Sylvester O'Halloran Surgical Scientific Meeting, March 2006

Parallel gene and protein expression analysis of the immune response to major surgery in oesophageal cancer patients treated with surgery alone or a multimodality regimen

Irish Society of Gastroenterology winter meeting, November 2005

Parallel gene and protein expression analysis of the immune response to major surgery in oesophageal cancer patients treated with surgery alone or a multimodality regimen

Sir Peter Freyer Surgical Symposium, September 2005

Parallel gene and protein expression analysis of the immune response to major surgery in oesophageal cancer patients treated with surgery alone or a multimodality regimen

Sylvester O'Halloran Surgical Scientific Meeting, March 2005

Inhibition of cox-2 and Oxidative pathways as a means to decrease oesophageal injury in a surgical reflux model

Irish Society of Gastroenterology winter meeting, November 2004

Failure of Vitamin C and COX-2 inhibitors to protect against oesophageal inflammation in an experimental model of severe reflux

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Chapter 1

Introduction

Part A   Animal studies

1.1 Aetiology of oesophageal cancer and Barrett's oesophagus

The incidence of oesophageal adenocarcinoma has increased markedly in the western world (Devesa et al., 1998). Barrett's oesophagus, which can be defined as columnar metaplasia of the distal oesophagus associated with chronic gastro-oesophageal reflux disease (Cameron, 2001), is a precursor to developing oesophageal cancer. Clinically, it is estimated that approximately 1/ 100 adults with regular reflux symptoms have Barrett's oesophagus (Cameron, 2002); approximately one in five adults complain of at least weekly reflux symptoms (Nebel et al., 1976), and endoscopy in those complaining of weekly heartburn demonstrates Barrett's (>3cm with intestinal metaplasia) in 5% of patients (Winters et al., 1987). Oesophageal adenocarcinoma varies in incidence in different populations and countries. In the USA, it occurs 4 times more commonly in white males than black males, and 8 times more commonly in white males than white females (Blot et al., 1991). In addition the incidence for males in different European countries varies greatly ranging from 0.5/ 100 000/ year in the Czech Republic to 6.9/ 100 000/ year in the United Kingdom (Cameron, 2002). The incidence for males in Ireland is especially high at 11.8/ 100 000/ year (National_Cancer_Registry_Ireland, 2007). Some of the major environmental factors associated with oesophageal adenocarcinoma include obesity and smoking. The Rate of oesophageal adenocarcinoma in the heaviest quarter of a Swedish population was almost 8 times that of the lightest quarter of the population (Lagergren et al., 1999). In the USA, a study by the National Cancer Institute found that smoking was associated with a greater that doubling of the risk of oesophageal adenocarcinoma (Gammon et al., 1997). Investigating the aetiology of and potential treatments for Barrett's oesophagus and oesophageal adenocarcinoma has involved the use of large animal, small animal and human models and these are described in more detail in the following sections.

1.2 The use of large animal models to investigate gastro-oesophageal reflux and Barrett's oesophagus

The main large animal used in studies of gastro-oesophageal reflux and Barrett's oesophagus has been the Dog. Relatively few experimental groups have used dogs because of their larger size and greater expense. In addition, a different application is necessary when applying for an animal licence to perform experimentation on dogs or cats. In 1968, Hennessy created a surgical mucosal defect in the lower canine oesophagus and performed a procedure to allow gastro-oesophageal reflux (Hennessy et al., 1968). However, when animals were sacrificed at a later stage, the mucosal defect had healed with squamous epithelium; therefore this did not produce a model of Barrett's oesophagus. In 1970, Bremner also dissected off the lower oesophageal mucosa in a canine model (Bremner et al., 1970). In animals with a competent lower oesophageal sphincter, the denuded area healed with squamous epithelium. However, when the lower oesophageal sphincter was destroyed and histamine administered to increase acid production, the denuded area became covered with columnar cells. Therefore Bremner concluded that the reflux of gastric acid produces Barrett's because of "Creeping Substitution" by gastric columnar cells. In 1988, Gillen formed a mucosal defect in the lower canine oesophagus, leaving a 1cm length of normal squamous mucosa below it (Gillen et al., 1988). A reflux inducing procedure was performed (Wendel cardioplasty and hiatus hernia), and animals were subjected to acid hyperstimulation for 3 months. This resulted in the mucosal defect healing with columnar epithelium. This was presumed to have developed from the normal oesophagus, probably from the submucosal glands in the oesophagus. In 1994, Li formed a mucosal defect again in the lower canine oesophagus; a reflux procedure was performed and animals were subjected to pentagastrin acid stimulation (Li et al., 1994). After 3 months, the healed mucosal defect was excised and this had repaired with columnar epithelium in 7/ 10 dogs. In 6 dogs, following the new mucosal defect, the reflux inducing surgery was reversed and dogs were treated with the proton pump inhibitor, omeprazole for 3 months. In all 6 dogs the defect had healed with columnar epithelium; however islands of squamous epithelium were also present. It was concluded that the type of regeneration depends on the depth of the original injury and that where both squamous and columnar epithelium are both present, they can both regenerate together; however columnar regeneration is predominant because of its greater turnover. This experiment also could be considered to have shown a partial regression of Barrett's oesophagus with antireflux surgery and better acid control.

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1.3   The use of Rodent models to investigate oesophageal cancer and Barrett's oesophagus

1.3.1 Normal anatomy of the rat oesophagus, stomach and duodenum

The anatomy of the gastrointestinal tract of the rat is broadly similar to humans. Like humans it contains an oesophagus, a stomach, a small intestine consisting of duodenum, jejunum and ileum and a large intestine consisting of a caecum (but without an appendix), colon and rectum (DeSesso and Jacobson, 2001, Suckow et al., 2006).

Unlike humans, the gastroesophageal junction in the rat does not occur at the same level as the diaphragmatic hiatus (Soto et al., 1997). The gastroesophageal junction is widely separated from the hiatus by a length of intra-abdominal oesophagus. This produces an enhanced barrier to reflux and is one reason of the reasons why rats do not vomit (Suckow et al., 2006, DeSesso and Jacobson, 2001). For the laboratory investigator, this also means that the oesophagus is easily accessible below the diaphragm, permitting the relatively straightforward formation of a oesophago-intestinal anastomosis (Suckow et al., 2006).

The rat stomach is a single chamber like the human stomach. The oesophagus enters the stomach more centrally in the rat than the human where it enters in an eccentric position near the fundus. In the rat, similar to all rodents, the stomach is made up of two distinct regions which can be seen macroscopically (figure 1.1). The stratified squamous epithelial lined forestomach holds food exiting from the oesophagus and allows bacterial digestion to commence. This is separated from the glandular stomach by the prominent limiting ridge. This three dimensional structure closes the orifice to the oesophagus during retching, another reason why reflux and vomiting do not normally occur in rats (DeSesso and Jacobson, 2001, Suckow et al., 2006). The glandular stomach has a secretory lining which secretes acid and proenzymes, similar to the entire stomach in humans.

Figure 1.1 Anatomy of the rat oesophagus and stomach
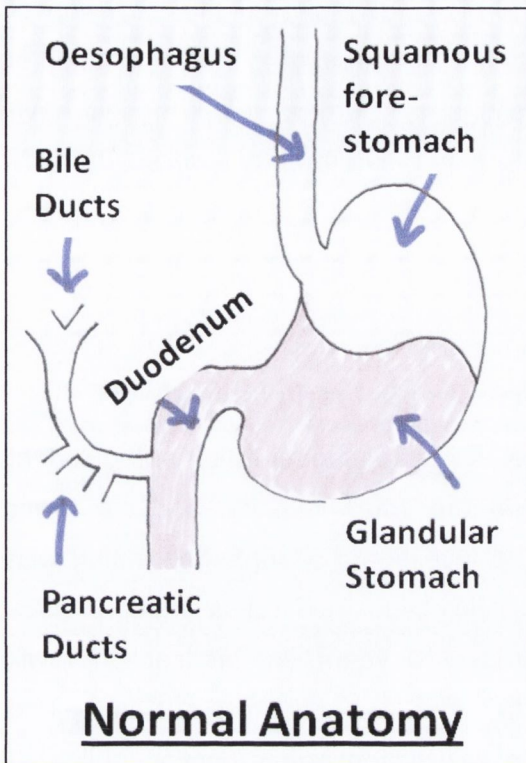
The duodenum in rats exits the stomach in a more caudal position than humans. The pancreatic and bile ducts enter into the duodenum. It is approximately 10cm long making up 8% of the rat's small intestinal length compared 4% in humans (DeSesso and Jacobson, 2001). This is convenient for the laboratory investigator forming reflux models with the rat duodenum.

1.3.2 Introduction to rodent models of oesophageal reflux

The short term and long term effects of oesophageal reflux injury have been studied in rats. Models investigating the short term reflux have involved duodenojejunal ligation (Wetscher et al., 1995), or blocking the duodenum using a ring (Oh et al., 2001) to prevent stomach emptying. This causes the eventual reflux of stomach contents into the oesophagus producing oesophageal injury. These short term studies necessitate the killing of animals at 24 hours on humane grounds and have allowed investigators to study the role of inflammation and oxidative stress in oesophageal reflux injury.

The effects of oesophageal reflux in rats have also been investigated in numerous studies in which a surgical anastomosis between the small intestine and the lower oesophagus has been formed. The surgical anastomosis models have allowed the effects of oesophageal reflux to be studied over a prolonged period of time during which inflammation, dysplasia and adenocarcinoma have been produced. The histological and

molecular changes occurring at different time periods after reflux-inducing surgery have been described. Variations in the operations performed have allowed the exclusion of components of reflux i.e. acid, bile or pancreatic enzymes to investigate the contribution of each towards the production of dysplasia and malignancy. Surgical reflux models have also been employed to investigate strategies which may prevent reflux induced Barrett's oesophagus and adenocarcinoma e.g. the use of non steroidal anti-inflammatory drugs (NSAIDS) and antioxidants.

1.3.3 Commonly used surgical models of oesophageal reflux in rodents

As described above (section 1.3.2), surgical models of oesophageal reflux in the rodent involve the formation of an anastomosis between the small intestine and the lower oesophagus. They have been used for 45 years to investigate oesophageal reflux with numerous models and modifications of these described. The reflux models have undergone considerable modification during the last 10 years. In this section I will describe four surgical reflux procedures. In the oesophagojejunostomy and oesophagoduodenostomy models (figure 1.2), the gastroesophageal junction is ligated with anastomosis of the lower oesophagus to small intestine, thus excluding stomach function. The more recently described oesophagogastroduodenal anastomosis and jejunum-oesophagogastric junction anastomosis (figure 1.3) involve the anastomosis of the gastroesophageal junction to the small intestine, with retention of stomach function.

Figure 1.2 The oesophagojejunostomy (A) and oesophagoduodenostomy (B) reflux procedures

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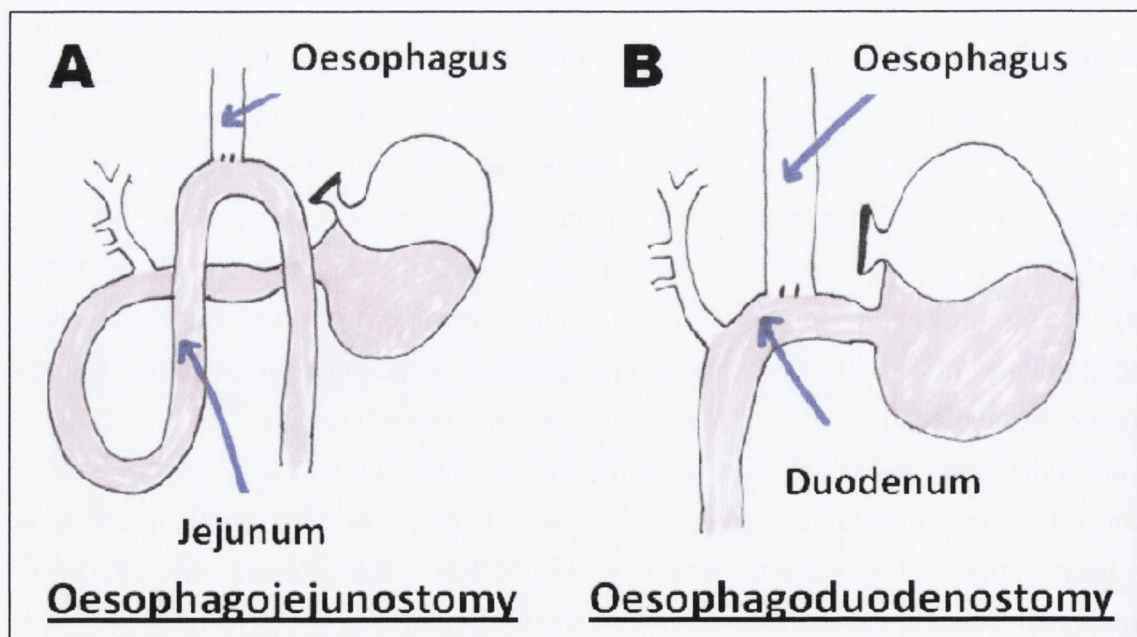
1.3.3.a   Oesophagojejunostomy

The oesophagojejunostomy reflux model was first described in 1962 (Levrat et al., 1962). This described oesophagitis caused by the reflux of duodenal contents into the oesophagus. The Levrat oesophagojejunostomy model has been extensively used by investigators since its description (Buttar et al., 2002, da Costa et al., 1993, Fein et al., 1998, Fein et al., 2000a, Fein et al., 2000b, Miwa et al., 1996, Pera et al., 1993, Pera et al., 1989, Van Den Boogert et al., 1999). This model results in the reflux of duodenal contents containing acid, bile and pancreatic juice into the oesophagus. It is important to note that although the stomach is retained and produces acid, it is defunctioned; i.e. food does not pass through it. This means its storage ability is lost and this results in malnutrition in animals. Modifications to this model have included the addition of carcinogen or iron overload after surgery, or the addition of a gastrectomy to the procedure and are described in detail below (section 1.3.4).

1.3.3.b   Oesophagoduodenostomy

The oesophagoduodenostomy reflux model is similar to the Levrat model and also results in the reflux of acid, bile and pancreatic juice into the oesophagus. Like the oesophagojejunostomy model, it has also been extensively investigated (Attwood et al., 1992, Goldstein et al., 1997, Clark et al., 1994, Goldstein et al., 1998, Ireland et al., 1996, Jang et al., 2004, Melo et al., 1999, Oberg et al., 2000, Chen et al., 2000b). Once again, the stomach produces acid but is defunctioned resulting in malnutrition. Like the Oesophagojejunostomy model, carcinogen use and dietary modification have been investigated and are described below (section 1.3.4).

Figure 1.3   The oesophagogastroduodenal anastomosis (A) and jejunum-oesophagogastric junction anastomosis (B) reflux procedures

Gastro-oesophageal Junction — **A**

Gastro-oesophageal Junction — **B**

Duodenum

Jejunum

<u>Oesophago-gastroduodenal Anastomosis</u>

<u>Jejunum-oesophagogastric Junction anastomosis</u>
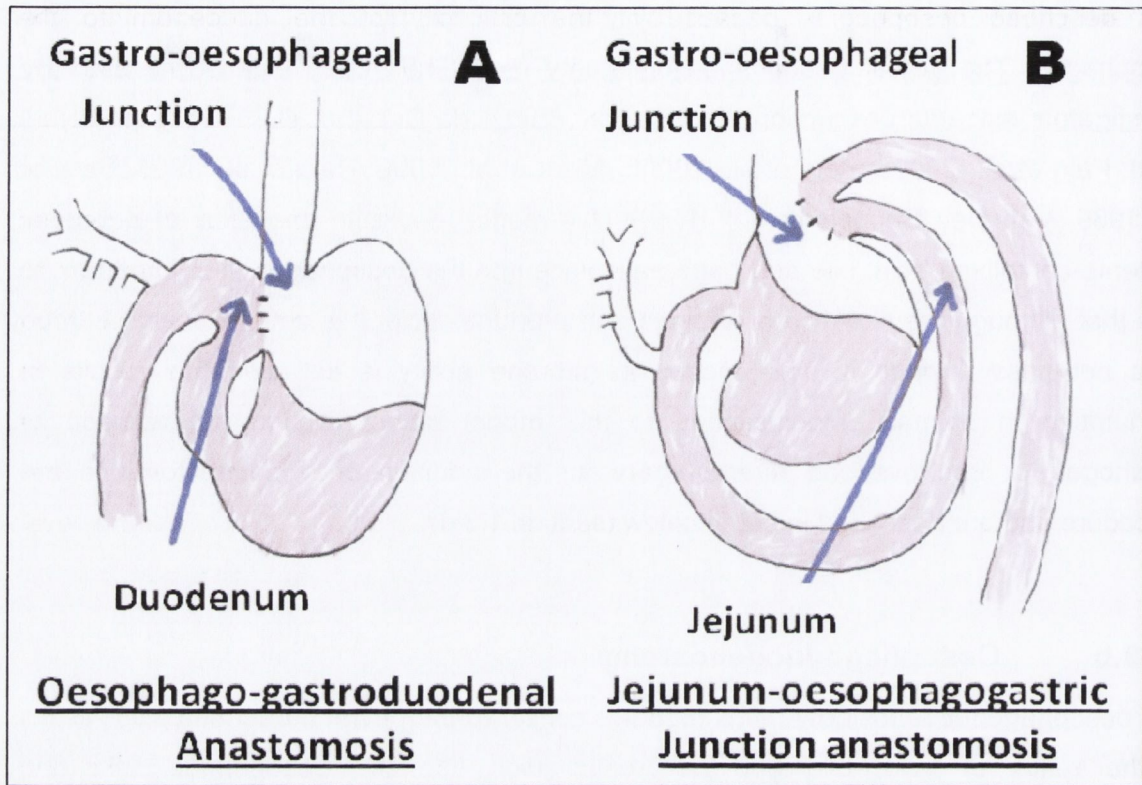
1.3.3.c   Oesophagogastroduodenal anastomosis

In the last ten years, investigators have sought to produce models of oesophageal reflux in the rat with preservation of stomach function to avoid malnutrition. The aim of this is to allow the study of reflux for more prolonged periods because the animals are healthier and thriving compared to the nutritionally deficient animals previously studied.

In 1999, an oesophagogastroduodenal anastomosis was described (Chen et al., 1999). This results in the reflux of duodenal and gastric contents into the oesophagus as in the previous models. Anastomosis to the gastroesophageal junction allows disruption of the lower oesophageal sphincter, and retention of the stomach attempts to minimise the alteration in gastrointestinal function. Interestingly, a similar surgical technique was described several years previously as a method of creating a oesophagoduodenostomy (Attwood et al., 1992). Chen's model has allowed the study oesophageal reflux up to 40 weeks postoperatively because of the animals enhanced nutritional state (Chen et al., 2002b).

1.3.3.d   jejunum-oesophagogastric junction anastomosis

The oesophagogastroduodenal anastomosis reflux model is admired because it retains stomach function. However because of the proximity of the duodenum to the gastroesophageal junction, anastomosis of these together may result in the stomach being curled up. This may deform the stomach reducing its volume and therefore lessening its function. To preserve the stomach with maintenance of its shape and function, a jejunum-oesophagogastric junction anastomosis has been recently described (Kumagai et al., 2003). In addition, the authors feel that the duodenal reflux produced in this model may closer resemble naturally occurring reflux in humans. This novel reflux model has been used by its authors to examine oesophageal reflux to 70 weeks postoperatively (Kumagai et al., 2004). A recent publication has compared the oesophagoduodenostomy model with gastrectomy to an oesophago-gastrojejunostomy model (Buskens et al., 2006). The oesophago-gastrojejunostomy they describe however is closer in nature to Chen's model than that described by Kumagai. In this study, animals are studied to 52 weeks postoperatively.

1.3.4 Oesophageal histological changes as a result of duodenal reflux and the effects of carcinogen and iron treatment.

The basic principle of the rat models of reflux is that they should produce similar effects to oesophageal reflux in humans. The models try to reproduce the inflammation, metaplasia, dysplasia and adenocarcinoma sequence observed in humans (figure 1.4). Indeed normal oesophagus progressing to inflammation, Barrett's and adenocarcinoma following the surgical creation of reflux have been found in numerous rat models of reflux (table 1.1). Rat models are therefore representative of reflux induced adenocarcinomas in humans.

Figure 1.4   The inflammation, metaplasia, dysplasia and adenocarcinoma sequence as a result of oesophageal reflux in humans and animal models of reflux
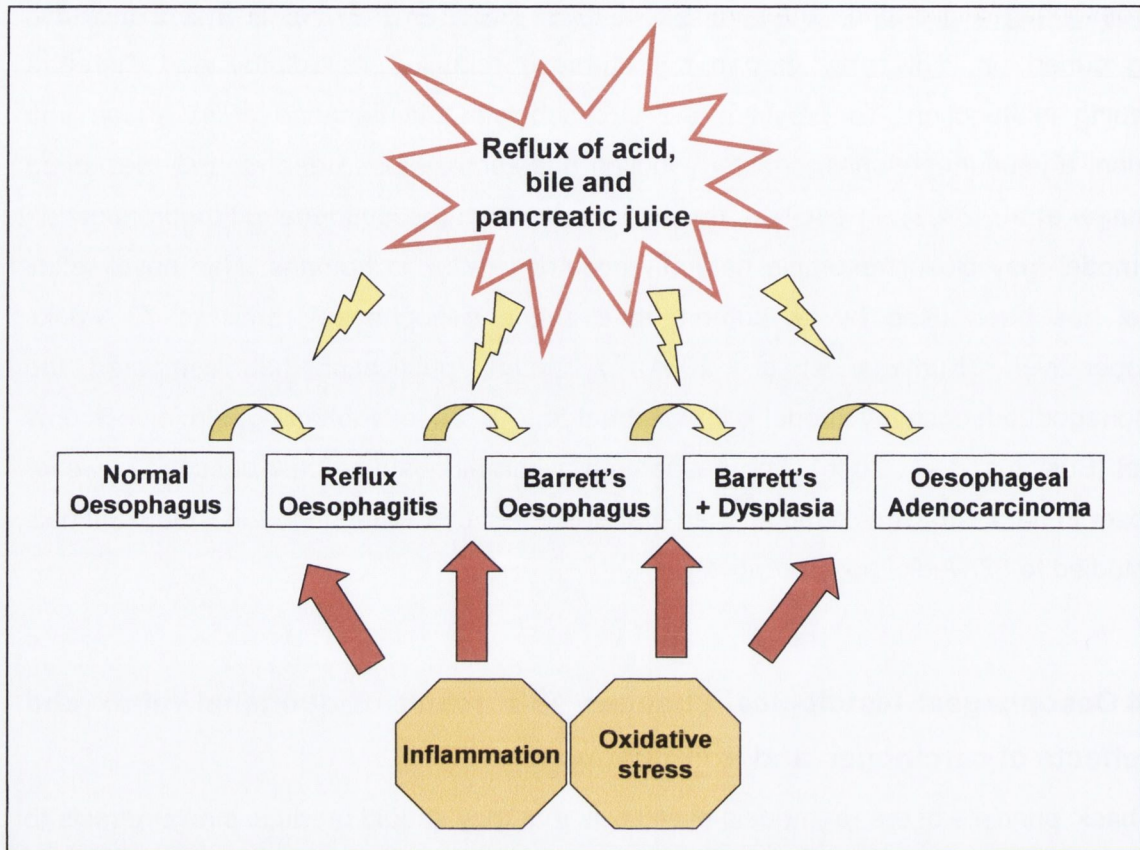
Table 1.1   Summary of the histological findings obtained after surgical reflux procedures in the rat. The presence and prevalence of oesophagitis, Barrett's oesophagitis and adenocarcinoma obtained in published experimental studies are shown, classified according to the reflux procedure performed.

Reflux model and study	Oesophagitis	Barrett's	Adenocarcinoma
Oesophagojejunostomy			
(Levrat et al., 1962)	+	-	-
(Pera et al., 1993)	+ 80-100% (24 weeks)	- 0% (24 weeks)	- 0% (24 weeks)
(Miwa et al., 1996)	+ 100% (50 weeks)	+ 100% (50 weeks)	+ 75% (50 weeks)
(Fein et al., 1998)	+ 100% (16 weeks)	+ 91% (22 weeks)	+ 55% (22 weeks)
(Buttar et al., 2002)	+ 100% (+iron, 23 weeks)	+ 80% (+iron, 28 weeks)	+ 51% (+iron, 28 weeks)
Oesophagoduodenostomy			
(Attwood et al., 1992)	+ 100% (30 weeks)	+	+ 7% (30 weeks)
(Ireland et al., 1996)	+ 72% (+carcinogen, 28 wks)	+ 10% (+carcinogen, 28 wks)	+ 30% (+carcinogen, 28 wks)
(Fein et al., 1998)	+ 100% (22 weeks)	+ 91% (22 weeks)	+ 55% (22 weeks)
(Goldstein et al., 1998)	+ 100% (+iron, 31 weeks)	+ 91% (+iron, 31 weeks)	+ 73% (+iron, 31 weeks)
(Melo et al., 1999)	+ 66.6% (22 weeks)	+ 16.7% (22 weeks)	+ 16.7% (22 weeks)
(Oberg et al., 2000)	+ 95% (28 weeks)	- 0% (28 weeks)	+ 20.3% (28 weeks)
(Jang et al., 2004)	+ 100% (40 weeks)	+ 100% (40 weeks)	+
Oesophago-gastroduodenal anastomosis			
(Chen et al., 1999)	+ 100% (-iron, 40 weeks); 100% (+iron, 40 weeks)	+ 53.5% (-iron, 40 weeks); 78.0% (+iron, 40 weeks)	+ 25.6% (-iron, 40 weeks); 53.7% (+iron, 40 weeks)
Jejunum-oesophagogastric junction anastomosis			
(Kumagai et al., 2003)	- 100% (50 weeks)	- 100% (50 weeks)	- 37.5% (50 weeks)

1.3.4.a   Reflux Oesophagitis

Reflux oesophagitis, the first step in the progression from normal oesophagus to adenocarcinoma is well described in all four animal models described above (section 1.3.3). The rates of oesophagitis produced in each of the reflux models (without the use of carcinogen or Iron overload) are discussed below.

The original oesophagojejunostomy model describes oesophagitis as a result of the reflux of duodenal contents (Levrat et al., 1962). In another oesophagojejunostomy model, oesophagitis was present macroscopically in 69% of animals at 16 weeks post-op (Fein et al., 1998). Microscopically, all 22 animals had evidence of oesophagitis which was moderate to severe in 82% of animals, with ulceration present in 77%. Similarly, oesophagitis has been produced following oesophagoduodenostomy with 61% of animals demonstrating marked oesophagitis 20 weeks following surgery in one example of this model (Melo et al., 1999). In Chen's oesophagogastroduodenal anastomosis model, all animals had evidence of oesophagitis 40 weeks post-op with an average oesophagitis grade of 1.7 (moderate) (Chen et al., 1999). The jejunum-oesophagogastric junction anastomosis model has been shown to cause oesophagitis in 8/12 of animals at 20 weeks post-op, with all 8 animals demonstrating oesophagitis from 30- 50 weeks post op (Kumagai et al., 2003).

1.3.4.b   Barrett's Oesophagus

Barrett's oesophagus is the presence of columnar metaplasia of the distal oesophagus associated with chronic gastroesophageal reflux disease (Cameron, 2001). The term "Barrett's ulcer" was first described in 1953 (Allison and Johnstone, 1953); the history of the term Barrett's oesophagus is interesting and has recently been summarised (Cameron, 2001).

Barrett's oesophagus has been described with some variability in each of the four rat surgical reflux models described (section 1.3.3), in animals not receiving carcinogen or Iron treatment. In an oesophagojejunostomy model, oesophageal columnar lining was found in 91% of animals at 16 weeks post-op (Fein et al., 1998). Miwa similarly found Barrett's in 100% of animals 50 weeks following oesophagojejunostomy (Miwa et al., 1996). Some investigators have had different findings: Pera "despite thorough dissection" was unable to find Barrett's in oesophagojejunostomy animals at 32 weeks post-op (Pera et al., 1993). Using the oesophagoduodenostomy reflux model, Barrett's was not present in some studies (Attwood et al., 1992, Oberg et al., 2000), present in low numbers in others (Ireland et al., 1996, Melo et al., 1999), and found in 86% of animals, starting as early as 10 weeks in another study (Jang et al., 2004). In this last study, atypical Barrett's oesophagus was found in 3/8 animals at 30 weeks and 3/7 animals at 40 weeks post-op

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(Jang et al., 2004). In the oesophagogastroduodenal anastomosis reflux model, Barrett's has been described as occurring in 53.5% of animals 40 weeks post-op with dysplasia occurring in 34.9% of rats. In a similar model described by Buskens, 1-2 mm of oesophageal columnar epithelium was found at the anastomosis in 10/10 animals at 16 weeks post-op, and a median length of 10mm oesophageal columnar epithelial lining was found in 9/10 animals 52 weeks post-op (Buskens et al., 2006). Dysplasia was not found in any of these animals. In the jejunum-oesophagogastric junction anastomosis model, specialised oesophageal columnar epithelial lining was described in 5/12 animals at 20 weeks post-op, 6/8 animals at 30 and 40 weeks post-op and in 8/8 animals at 50 weeks post op (Kumagai et al., 2003). Dysplasia was not found in these rats.

The occurrence of Barrett's in rodent reflux models is controversial. Goldstein recognised that the definition of Barrett's used in animal models is important and advised not to describe the presence of puckered small bowel mucosa in the oesophagus because of healing as Barrett's (Goldstein et al., 1997). Oberg et al suggested that columnar lining in the oesophagus could be an artefact caused by the method of embedding in paraffin and sectioning or perhaps related to the implantation of columnar epithelium by sutures (Oberg et al., 2000). They argue that admixed squamous and columnar epithelium at the anastomosis used in some models to describe Barrett's, should be interpreted with care. Buskens et al admit that while a 1-2 mm length of columnar epithelium in the lower oesophagus of many animals resembled a Barrett's segment, this could not be confirmed because the location and short length meant that suture implantation or jejunal overgrowth could not be outruled (Buskens et al., 2006).

1.3.4.c   Oesophageal adenocarcinoma

Oesophageal carcinoma occurs in animal reflux models when dysplastic epithelium (with abnormal cell polarity, maturation, nuclear atypia and mitotic figures) is observed along with invasion through the basement membrane (Buttar et al., 2002). The carcinomas occurring in animal reflux models may be classified as adenocarcinoma, squamous cell cancer or adenosquamous cancer. Oesophageal carcinomas have been found in the described reflux models (section 1.3.3) without using carcinogen or iron supplementation.

In an oesophagojejunostomy model, 55% of animals were found to have oesophageal adenocarcinoma, 16 weeks after surgery (Fein et al., 1998). In another oesophagojejunostomy model, 10/12 rats (83%) of rats were found to have carcinomas, 50 weeks post-op. nine of these carcinomas were adenocarcinoma, 2 were squamous cell and 2 were adenosquamous (Miwa et al., 1996). Using the oesophagoduodenal anastomosis, the rates of oesophageal carcinoma vary: In one model only 1/14 rats (7%) demonstrated oesophageal adenocarcinoma 22 weeks post-op. In another, tumours were

found at the anastomosis in 14/59 rats (23.7%), 28 weeks post-op; 12/59 (20.3%) of these were adenocarcinomas (Oberg et al., 2000). In the oesophagogastroduodenal anastomosis, 11/43 rats (25.6%) developed oesophageal adenocarcinoma 40 weeks post-op (Chen et al., 1999). Using the jejunum-oesophagogastric junction anastomosis model, adenocarcinoma was found in 1/8 rats at 40 weeks and 3/8 rats at 50 weeks post-op. In addition, 1/8 rats demonstrated the presence of an adenosquamous carcinoma at 50 weeks (Kumagai et al., 2003).

Like Barrett's, the oesophageal carcinomas produced in rat reflux models are controversial. In an esophagogastrojejunostomy model, tumours histologically resembling adenocarcinoma were found in 7/10 rats 52 weeks post-op (Buskens et al., 2006). However the authors found no evidence of precursor lesions to these tumours. Immunohistochemical evidence of malignancy examining p53 mutation and a significant increase in the proliferation index was not found. In addition the authors commented that the "adenocarcinoma" reported previously in the literature were mostly well differentiated and limited to the submucosa. They concluded that these tumours most likely develop as a result of mechanical forces (e.g. sutures) transposing mucosal glands into the submucosa rather than being induced by duodeno-gastroesophageal reflux. The tumours more resemble "Oesophagitis cystica profunda" an inflammatory condition which in humans does not develop into malignancy. Buskens et al therefore place in doubt the usefulness of the animal model of reflux in investigating true adenocarcinoma developing in a Barrett's segment of oesophagus.

1.3.4.d   The use of carcinogens and iron overload

Carcinogens were commonly used in rodent models of reflux until approximately 10 years ago. Compounds used included nitrosamines e.g. methyl-n-amylnitrosamine (MNAN) (Attwood et al., 1992) and diethylnitrosamine (DEN) (Melo et al., 1999). The reason for using these compounds was to mimic the carcinogenic effects of cigarette smoking (Mirvish, 1997). The use of carcinogens in animal reflux models has fallen out of vogue. The administration of carcinogens has become difficult to justify on ethical grounds and in addition the findings produced with their use do not add to the understanding of reflux induced oesophageal adenocarcinoma.

Oesophageal carcinoma may be produced in animals following the administration of carcinogen alone without the need for surgically induced reflux. Using Wistar rats, carcinomas were found in 19/20 animals that received diethylnitrosamine (DEN) in drinking water for 120 days (Sallet et al., 2002). The tumours produced were squamous cell carcinomas and multiple tumours (mean of 6 per animal) were found in each animal. The effect of carcinogen administration on the degree of oesophagitis and rate of Barrett's

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in surgical reflux models are poorly reported. In an oesophagoduodenostomy study the rate of oesophagitis was 23.5% in the surgery and carcinogen (DEN) group compared to 61.1% in the surgery only group at 20 weeks (Melo et al., 1999). In the same study, Barrett's was found in none of the animals treated with surgery and DEN, but found in 16.7% of those treated with surgery alone. The use of carcinogens in rodent surgical reflux models increases the yield of carcinomas which are mixed in nature (i.e. squamous cell carcinoma and adenocarcinoma). In an experiment using 2, 6-dimethylnitrosomorpholine (DMNM), no tumours were found in control animals and 25% of animals that received DMNM developed oesophageal squamous cell carcinomas. In animals that underwent oesophagoduodenostomy, 7% developed oesophageal adenocarcinoma and in those that underwent oesophagoduodenostomy followed by treatment with DMNM, 45% developed squamous cell carcinoma, 35% developed adenocarcinoma and one animal (5%) developed a rhabdomyosarcoma (Attwood et al., 1992). In another study using DEN as the carcinogen, no control animals or animals that received DEN treatment alone developed carcinomas. In those that underwent oesophagoduodenostomy 16.7% developed adenocarcinoma and in those treated with DEN following oesophagoduodenostomy, 76.5% developed carcinomas, of which one (5.9%) was an adenocarcinoma and 70.6% were epidermoid (squamous cell) carcinomas (Melo et al., 1999). In summary, the use of carcinogens in surgical reflux models has a varying effect on the rates of oesophagitis and Barrett's but increases the yield of carcinomas. The carcinomas produced are mixed in nature, mostly squamous cell, unlike the adenocarcinomas produced in the human reflux model of cancer. Therefore animal models using carcinogens are difficult to interpret and probably redundant.

As the use of carcinogens in the rat model of reflux has declined in popularity, the use of iron overload has increased. It was recognised 10 years ago that animals surgically treated to produce reflux developed anaemia, possibly due to iron absorption (Goldstein et al., 1997). The authors used iron supplementation in animals following oesophagoduodenostomy and produced oesophageal adenocarcinoma in 73% of animals after 31 weeks in comparison to 54% of animals who received surgery, iron and N'-Nitrosonornicotine (NNN) carcinogen. Following this, they studied the effects of iron supplementation versus none in an oesophagoduodenostomy model. Animals treated with surgery and Iron compared to those treated with surgery alone had more severe oesophagitis, greater levels of cell proliferation, inducible nitric oxide synthase (iNOS) and nitrotyrosine and enhanced tumourigenesis (73% versus 0% at 31 weeks) (Goldstein et al., 1998). This theme was further investigated in an Oesophagogastroduodenal anastomosis model: animals treated with intraperitoneal iron supplementation developed increased rates of Barrett's, Barrett's with dysplasia and adenocarcinoma than those treated with surgery alone (78%, 53.7% and 53.7% versus 53.5%, 34.9% and 25.6%)

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(Chen et al., 1999). Another study demonstrated that animals treated with iron supplementation following oesophagoduodenal anastomosis had evidence of oesophageal iron overload, and that oxidative damage to DNA, protein and lipid in the oesophagus was significantly higher than in non operated controls (Chen et al., 2000a).

In summary, animal reflux models supplemented with iron overload result in enhanced Barrett's formation and tumourigenesis by increasing oesophageal inflammation and oxidative stress. The authors of the above studies feel that iron overload may have a role in explaining oesophageal adenocarcinoma in humans. Increased rates of oesophageal cancer in those with haemochromatosis may be evidence of this (Hsing et al., 1995). They feel that iron over-nutrition in humans because of excess consumption of iron in red meat, especially in males may be a risk factor for oesophageal adenocarcinoma.

1.4 Evidence in humans; Epidemiology and prevention of Barrett's oesophagus and oesophageal adenocarcinoma

As discussed above (section 1.1), oesophageal adenocarcinoma has increased greatly in incidence. This increase has occurred mainly in European males and in American white males and increased oesophageal adenocarcinoma is seen in smokers and the obese. Therefore, we can see from epidemiological evidence, two population measures to reduce the incidence of oesophageal adenocarcinoma are to reduce smoking and obesity rates.

The effects of diet and pharmacological treatments have been examined in humans as possible ways to reduce oesophageal adenocarcinoma. Proton pump inhibitors (PPIs) are a standard treatment strategy in trying to preventing Barrett's related oesophageal cancer through acid suppression. However Sampliner (Sampliner, 1994) failed to show that high dose treatment significantly shortens the length of Barrett's in humans, placing doubt on the validity of chemoprevention using PPIs. Anti-inflammatories, frequently employed in experimental animal models have been examined in human epidemiological studies. Their value in preventing oesophageal cancer has been questioned in an extensive epidemiological study in which Non-aspirin NSAID long-term use was associated with a reduced risk of gastric cancer, but their role in preventing oesophageal cancer could not be established (Lindblad et al., 2005). Coupled with safety concerns about COX-2 inhibitors (Couzin, 2005), there is doubt about the usefulness of these agents in the chemoprevention of oesophageal cancer. The role of diet, vitamin and antioxidant intake in oesophageal adenocarcinoma has been established and may provide a target for prevention. Low intake of vitamins C and E has been found to correlate with the development of both types of oesophageal cancer in males (Bollschweiler et al., 2002). Swedish subjects with a high intake of vitamin C, beta-carotene, and alpha-tocopherol showed a 40-50% decreased risk of both histological types of esophageal cancer

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compared with subjects with a low intake (Terry et al., 2000). In Nebraska, in a case controlled study, a diet high in fruit and vegetables was associated with a reduced risk of esophageal adenocarcinoma (Chen et al., 2002a). Health promotion measures to encourage a diet high in fruit, vegetables and vitamins may prove effective in reducing oesophageal cancer rates. Population studies investigating dietary supplementation with vitamins and minerals have been performed. In Linxian, China, an area with a high incidence of oesophageal and stomach cancer, subjects supplemented over a 5 year period with beta carotene, vitamin E, and selenium had a lower death rate, cancer rate and especially stomach cancer rate than controls (Blot et al., 1993). Because antioxidants are inexpensive to produce and largely without side effects, they are attractive agents in preventing oesophageal adenocarcinoma.

A publication in the Journal of the National Cancer Institute concludes that a few known risk factors account for a majority of oesophageal and gastric cancers. The authors suggest that "the incidence of these cancers may be decreased by reducing the prevalence of smoking, gastroesophageal reflux, and being overweight and by increasing the consumption of fruits and vegetables" (Engel et al., 2003).

Part B   Human Studies

1.5   Multimodal therapy for oesophageal cancer

Oesophageal adenocarcinoma has increased markedly in incidence in western society (Devesa et al., 1998). The overall five year survival rate for patients with oesophageal cancer is 10% (Faivre et al., 1998). In addition, surgical treatment carries significant danger with up to a 14% mortality rate and a 60% risk of morbidity (McCulloch et al., 2003).

A multimodality treatment approach, combining chemotherapy and radiotherapy before surgery, is increasingly used in gastrointestinal cancers such as oesophageal cancer and rectal cancer. Multimodal therapy in oesophageal cancer has been investigated extensively in a number of randomized controlled clinical trials (Nygaard et al., 1992, Le Prise et al., 1994, Apinop et al., 1994, Walsh et al., 1996, Bosset et al., 1997, Urba et al., 2001) and several meta-analyses (Fiorica et al., 2004, Malthaner et al., 2004, Urschel and Vasan, 2003). These have concluded that preoperative chemoradiotherapy leads to pathological tumour down-staging and improved three year survival at a cost of increasing postoperative mortality (Fiorica et al., 2004, Malthaner et al., 2004, Urschel and Vasan, 2003). Meta-analysis findings must be interpreted with care because inadequacies in individual trials may have overall implications (Demeester, 2005), however they remain the best evidence available.

1.6   The immune response following surgery

1.6.1   Introduction

An oesophagectomy is a striking example of major controlled surgical trauma, with major endocrine, neuroendocrine, physiologic and metabolic changes, and a significant potential for postoperative immune cell and cytokine changes. These responses are principally mediated through the effects of hormones and cytokines (Yamauchi et al., 1998, Tashiro et al., 1999, Lin et al., 2000). The additive effects of a thoracotomy and laparotomy during an Oesophagectomy produce an immense response compared to most surgical procedures such as a gastrectomy (Tashiro et al., 1999) or mastectomy (Yamauchi et al., 1998). Regulation of the systemic inflammatory response after surgery involves a complex interplay between pro-inflammatory and anti-inflammatory cytokines. The systemic inflammatory response (SIRS) is defined as two or more of the following; a temperature

>38°C or <36°C, heart rate >90 beats per minute, respiratory rate > 20 breaths per minute and white cell count >12,000/mm^3 or <4,000/mm^3 (Bone et al., 1992). Recovery following major surgery is described as a period of systemic inflammatory response followed by a period of convalescence mediated by counter-regulatory anti-inflammatory response (CARS). If the initial injury results in severe inflammation, this may lead to acute multiple organ failure (MOF) and early death after injury. On the other hand, a lesser inflammatory response followed by excessive CARS may induce a prolonged immunosuppressed state that can also be fatal (Lin et al., 2000).

1.6.2 Cytokines and growth factors in the postoperative inflammatory response

Cytokines are small to medium sized proteins produced by various cells including B-Cells, T-Cells, macrophages, basophils, endothelial cells and mast cells. They communicate with each other and with other cells using a network of cytokines and growth factors (Figure 1.5) (Townsend and McKenzie, 2000). These regulate important cellular processes involved in the immune response e.g. chemotaxis, proliferation and cellular differentiation. A summary of the main cytokines and growth factors of interest in this thesis, their producing and target cells and their functions is included below (Table 1.2). The 12 cytokines and growth factors studied in detail are: interleukin 2 (IL-2), interleukin 4 (IL-4), interleukin 6 (IL-6), interleukin 8 (IL-8), interleukin 10 (IL-10), vascular endothelial growth factor (VEGF), interferon gamma (IFN-γ), tumour necrosis factor alpha (TNF-α), interleukin 1 alpha (IL-1α), interleukin 1 beta (IL-1β), monocyte chemoattractant protein 1 (MCP-1) and epidermal growth factor (EGF).

Fig 1.5   Cells involved in the immune response and their inter-communication using cytokines, adapted from (Qiagen, 2009).
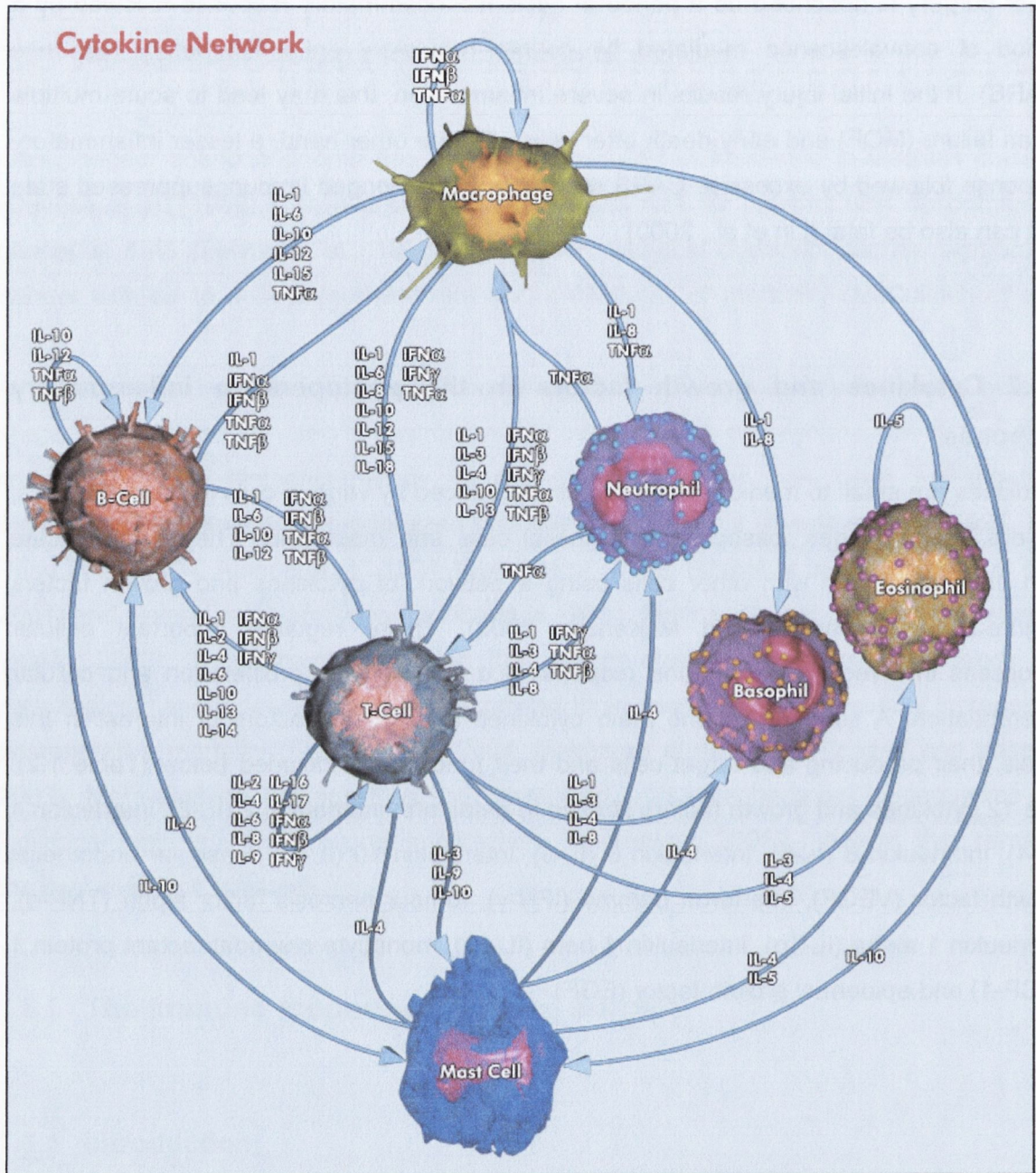
Table 1.2 **A summary of selected cytokines and growth factors, their producing cells, target cells and activities, adapted from (Decker, 2006).**

Selected Cytokines, Growth Factors and Their Activities			
Cytokine	Producing Cell	Target Cell	Function
IL-2	Th1 cells	activated T and B cells, NK cells	growth, proliferation, activation
IL-4	Th2 cells	activated B cells	proliferation and differentiation IgG, and IgE synthesis
		macrophages	MHC Class II
		T cells	proliferation
IL-6	monocytes	activated B cells	differentiation into plasma cells
	macrophages	plasma cells	antibody secretion
	Th2 cells	stem cells	differentiation
	stromal cells	various	acute phase response
IL-8	macrophages endothelial cells	neutrophils	chemotaxis
IL-10	Th2 cells	macrophages	*cytokine production*
		B cells	activation
VEGF	various	endothelial cells	Angiogenesis, vasodilatation
		macrophages	chemotaxis
IFN-γ	Th1 cells, Tc cells, NK cells	various	*Viral replication*
		macrophages	MHC expression
		activated B cells	Ig class switch to IgG$_{2a}$
		Th2 cells	*proliferation*
		macrophages	pathogen elimination
TNFα	macrophages, mast cells, NK cells	macrophages	CAM and cytokine expression
		tumor cells	cell death
IL-1α	monocytes	Th cells	co-stimulation
IL-1β	macrophages	B cells	maturation and proliferation
	B cells	NK cells	activation
	DC	various	inflammation, acute phase response, fever
MCP1	Various	monocytes	chemotaxis, inflammation, anti-apoptosis,
		T cells, basophils	cell adhesion
EGF	platelets, macrophages	various epidermal and epithelial	Cellular proliferation, differentiation and survival

1.6.2.a   Interleukin 10 (IL-10)

Interleukin 10 (IL-10) is a potent anti-inflammatory cytokine with a broad spectrum of action. It's role has been firmly established in various models of infection and inflammation (Mosser and Zhang, 2008). IL-10 is produced by a wide range of cells, ranging from T-cells and B-cells to epithelial and even tumour cells. Its main functions include inhibition of antigen presentation and inhibition of the production of pro-inflammatory cytokines and chemokines, e.g. IL-6, TNF-α and MCP-1. Serum or plasma IL-10 levels typically peak 24 hours following major surgery e.g. oesophagectomy (Sato et al., 2002, Sato et al., 2001, Yamaguchi et al., 2006, Yamauchi et al., 1998), abdominal aortic aneurysm repair (Bown et al., 2003) and liver resection (Kimura et al., 2006). Plasma IL-10 levels correlate with the extent of surgery; increases were found following oesophagectomy and pulmonary lobectomy, but not following mastectomy or laparoscopic cholecystectomy (Yamauchi et al., 1998). Increased plasma IL-10 levels have been associated with increased morbidity;

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following liver resection they correlated with postoperative infections (Kimura et al., 2006) and in severely injured patients detectable IL-10 was associated with the development of sepsis (Sherry et al., 1996). Mononuclear IL-10 mRNA expression levels have been examined following cardiac surgery with cardiopulmonary bypass; these were increased at 1 and 6 hours postoperatively. This increase was greater at both time-points in patients who did not require ionotropic support following surgery (more favourable) than those who did (Duggan et al., 2006). Whole blood IL-10 mRNA expression levels following ICU admission with sepsis have been investigated; in contrast to the previous study these were significantly up-regulated at 24 hours in patients who did not survive but not in survivors (Abe et al., 2007).

There are no published studies comparing IL-10 levels after surgery in patients treated with surgery alone versus a multimodal treatment protocol. Stimulated T cell IL-10 secretion in oesophageal cancer patients was previously found to remain unchanged before and 4 weeks following neoadjuvant CRT (Heidecke et al., 2002).

1.6.2.b Tumour necrosis factor alpha (TNF-α)

Tumour necrosis factor is considered by some as the principle pro-inflammatory cytokine (Duggan et al., 2006). Its actions include cell proliferation, survival and also apoptosis and are mediated by the activation of nuclear factor-kappaβ (NF-κβ) and mitogen-activated protein kinases (Gaur and Aggarwal, 2003). Its detection in plasma is difficult because of the affinity with which it binds to cell receptors. Plasma TNF-α levels were previously undetectable after oesophagectomy, gastrectomy, mastectomy and laparoscopic cholecystectomy (Yamauchi et al., 1998) and levels remained unchanged following abdominal aortic aneurysm repair (Bown et al., 2003). In patients treated with surgery alone for rectal cancer, serum TNF-α levels increased on pod 5 (Wichmann et al., 2003). In patients treated with preoperative CRT, serum TNF-α levels previously remained unchanged during CRT for oesophageal cancer (Zemanova et al., 2005), and were unchanged after CRT and after surgery for rectal cancer (Wichmann et al., 2003). TNF-α mRNA expression levels have been examined following cardiac surgery with cardiopulmonary bypass; Whole blood expression at 6 hours postoperatively (Zimmermann et al., 2003), and PBMC expression at 1 and 6 hours (Duggan et al., 2006) postoperatively have been found to be significantly reduced. The reduction in PBMC TNF-α expression was greater at both time-points in patients who did not require ionotropic support following surgery (more favourable) than those who did (Duggan et al., 2006).

Postoperative serum TNF-α levels following rectal cancer surgery were previously found to be significantly higher on pod 1, pod 2 and pod 5 in patients treated with surgery alone compared to those treated with preoperative CRT (Wichmann et al., 2003). There are no

published trials examining TNF-α levels following oesophagectomy in patients treated with or without pre-op CRT.

The ratio of IL-10: TNF-α mRNA expression has been used to provide a measure of the level of anti-inflammatory compared to pro-inflammatory cytokine expression in whole blood and peripheral blood mononuclear cells (PBMCs) (Duggan et al., 2006).

1.6.2.c Interleukin 6 (IL-6)

Interleukin 6 (IL-6) is an important pro-inflammatory cytokine involved in cell regulation and the regulation of the acute-phase response to injury and infection (Heinrich et al., 2003). It is produced by monocytes, macrophages, stromal cells, etc. and targets various cells including activated B-cells, plasma cells and stem cells. IL-6 has been broadly studied following major surgical procedures. Increased plasma and serum levels have been detected, with a peak at 6-24 hours postoperatively following oesophagectomy (Yamauchi et al., 1998, Tashiro et al., 1999, Sato et al., 2002, Takeda et al., 2003, Aiko et al., 2005, Narumiya et al., 2005, Fukunaga et al., 2001, Shibasaki et al., 2006), abdominal aortic aneurysm repair (Berguer et al., 1999, Bown et al., 2003), gastrectomy (Tashiro et al., 1999), pulmonary lobectomy (Yamauchi et al., 1998) and liver resection (Kimura et al., 2006). The magnitude of the IL-6 response following surgery correlates with the extent of surgery; levels following conventional oesophagectomy were significantly greater than those following thorascopic oesophagectomy (Fukunaga et al., 2001), oesophagectomy using a "mini-thoracotomy" (Narumiya et al., 2005), gastrectomy (Tashiro et al., 1999), cholecystectomy (Yamauchi et al., 1998, Yamaguchi et al., 2006) or mastectomy (Yamauchi et al., 1998).

A reduced serum IL-6 response has been described following rectal cancer surgery in patients treated with preoperative CRT (Wichmann et al., 2003). In contrast, an enhanced IL-6 response was reported following lung cancer resection in patients who received preoperative chemotherapy (Endo et al., 2004).

1.6.2.d Interleukin 8 (IL-8)

Interleukin 8 (IL-8) is a pro-inflammatory cytokine produced mainly by macrophages and endothelial cells, but also by tumour cells in response to chemotherapeutic intervention and hypoxia. Its main function is the promotion of neutrophil chemotaxis and degranulation (Waugh and Wilson, 2008). Significant increases in serum IL-8 levels have been described following major surgery e.g. oesophagectomy (Berguer et al., 2000, Fukunaga et al., 2001, Sato et al., 2001, Tsukada et al., 2001, Sato et al., 2002, Takeda et al., 2003, Aiko et al., 2005, Kimura et al., 2006). An increased IL-8 response correlates

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with the extent of surgery; levels were greater following transthoracic oesophagectomy than following thorascopic oesophagectomy (Fukunaga et al., 2001). Following oesophagectomy Il-8 levels have correlated with the occurrence of pulmonary complications (Tsukada et al., 2001); in addition concentrations in bronchoalveolar lavage fluid were 20 times greater than that in peripheral blood on pod 1 (Sato et al., 2001). Postoperative Il-8 levels following surgery in patients treated with or without neoadjuvant CRT have not been examined in previous studies to our knowledge.

1.6.2.e   Vascular endothelial growth factor

Vascular endothelial growth factor (VEGF) has an important role in targeting endothelial cells and results in vasculogenesis and angiogenesis (Lohela et al., 2009). In addition it has a role in the immune response and is chemotactic for macrophages and granulocytes and results in vasodilatation by releasing nitric oxide (NO). VEGF can be induced in a variety of cells which are not receiving enough oxygen, stimulated by hypoxia- induced factor (HIF). VEGF levels have previously been reported to be significantly raised on pod 5 and pod 10 following oesophagectomy; the authors found that although VEGF may arise from wound healing or be derived from tumour cells, activated platelets which produce VEGF were also increased postoperatively (Spence et al., 2002).

VEGF levels have been examined during the course of preoperative CRT for oesophageal cancer where no change in levels were detected (McDonnell et al., 2001). In the same study, VEGF levels in multimodal patients fell on pod 1 following oesophagectomy, returning to pre-op levels on pod 5. In this study, the post oesophagectomy VEGF response in multimodal patients was compared to the response in patients undergoing 6 varying procedures for non-malignant disease. To our knowledge, VEGF levels following oesophagectomy have not been directly compared in patients treated with or without neoadjuvant chemoradiotherapy.

1.6.2.f   Monocyte chemoattractant protein 1 (MCP-1)

Monocyte chemoattractant protein 1 (MCP-1), also known as Chemokine (C-C motif) ligand 2 (CCL2), is a potent chemoattractant for monocytes and macrophages (Melgarejo et al., 2009). MCP-1 is secreted by fibroblasts, endothelial cells, monocytes etc. and has an important role in conditions reliant on inflammation for progression including arthritis (Taylor et al., 2000) and cancer (O'Hayre et al., 2008). Increased MCP1 levels have been found to occur in the first 24 hours after major surgery, remaining raised for several days afterwards (Shibasaki et al., 2006, Kimura et al., 2006). The postoperative serum MCP1 response correlates with the severity of surgery and was greater following

oesophagectomy than following gastric or colorectal surgery (Shibasaki et al., 2006). Plasma MCP1 levels were higher in patients with organ dysfunction following liver resection (Kimura et al., 2006). The effect of chemoradiotherapy on MCP1 levels has not been studied before.

1.6.2.g   Epidermal growth factor (EGF)

Epidermal Growth Factor (EGF) is a growth factor produced by epidermal e.g. epithelial cells. It has an important role in inflammation and oncology, regulating cell growth, proliferation, differentiation and survival (D'Andrea and Gasparini, 2007). Serum EGF levels have been studied after liver resection for metastasis (de Jong et al., 2004) and also using biochip array technology following cataract surgery (Tu et al., 2007); in both studies, no post-op change in serum EGF levels were uncovered; however aqueous humour EGF levels increased in patients after cataract surgery (Tu et al., 2007). Levels have not been examined during or following chemoradiotherapy.

1.6.2.h   Interleukins 2 and 4 (IL-2 and IL-4), interferon gamma (IFN-γ) and interleukins 1 alpha and 1 beta (IL-1α and IL-1β)

Several other cytokines have an important role in the postoperative inflammatory response. These include interleukins 2, 4, interferon gamma and interleukins 1α, 1β (IL-2, IL-4, IFN-γ, IL-1α and IL-1β). Their functions are summarised in Table 1.2, including roles in cellular proliferation, activation and maturation. Relatively few studies have examined serum or plasma IL-2, IL-4 or IFN-γ levels following surgery. The plasma levels of IL-2 and IL-4 following cataract surgery (Tu et al., 2007) and IL-4 following liver resection (Kimura et al., 2006) have been examined and no change was found in the postoperative period. Small but significant changes in serum IFN-γ levels were detected 18 hours following cataract surgery (Tu et al., 2007). Postoperative production of these cytokines by stimulated peripheral blood mononuclear cells (PBMCs) has been investigated. Staphylococcal enterotoxin B (SEB) stimulated PBMC IL-2 production was found to increase significantly following major upper gastrointestinal surgery (Sweeney et al., 2005) and open abdominal aortic aneurysm repair (Sweeney et al., 2002) but not in patients diagnosed with acute pancreatitis (Sweeney et al., 2003). In a contrasting study, stimulated blood cell production of IL-2 was significantly lower 24 hours following transhiatal or transthoracic oesophagectomy (van Sandick et al., 2003). In this study stimulated IL-4 and IFN-γ production also fell on pod1 (van Sandick et al., 2003). In contrast, SEB stimulated PBMC IFN-γ production remained unchanged following major upper gastrointestinal surgery in an aforementioned study (Sweeney et al., 2005).

Serum or plasma IL-1β levels were previously undetectable following oesophagectomy, gastrectomy etc. (Yamauchi et al., 1998), and no significant change was demonstrated following aneurysm repair (Bown et al., 2003).

Postoperative changes in IL-1α levels have been infrequently studied; no change was found following cataract surgery (Tu et al., 2007).

Chapter 2

Aims and Objectives

2.1 Overall aims of the thesis

The title of this thesis is "local and systemic inflammation in oesophageal disease". The overall aims and objectives were to examine the role of inflammation in two settings in oesophageal disease.

Firstly, in an animal model of surgically induced reflux, we aimed to investigate the effects of local inflammation on oesophageal injury, possible tumour induction and strategies which may reduce these.

Secondly, in a human model of inflammation following major surgery for oesophageal cancer, we aimed to examine the systemic inflammatory cytokine response in detail at protein and RNA levels.

2.2 Specific aims of the animal studies

In chapter 3, our first aim was to establish an oesophagojejunostomy reflux model in the rodent. This involved consideration and optimisation of the choice of animal used, the preoperative preparation, the type of anaesthesia, the surgical technique, the recovery of animals following surgery and postoperative monitoring. We also aimed to choose a COX-2 inhibitor and an antioxidant to potentially reduce oesophageal injury and inflammation in the reflux model. We considered the dosage, method of administration, and proof of absorption of these medications.

In chapter 4, we aimed to investigate the degree of oesophageal inflammation in a rodent oesophagojejunostomy model, comparing animals undergoing surgery only (control animals) and those treated following surgery with COX-2 inhibitors or vitamin C. Additionally, we aimed to compare the percentage oesophageal ulceration determined by image analysis, animal weight and animal survival between groups.

2.3 Specific aims of the human studies

In chapter 5, our first aim was to perform a detailed analysis of the serum cytokine and growth factor response following oesophagectomy using "biochip" assay technology, in patients treated with surgery alone and patients treated with multimodal therapy i.e. combined chemoradiotherapy before surgery. We also aimed to compare the postoperative serum cytokine response following oesophagectomy in patients treated with

surgery alone or a multimodal treatment regimen, to examine for differences between these strategies.

In chapter 6 we aimed to examine whole blood and peripheral blood monocuclear cell (PBMC) IL-10 and TNF-α, mRNA expression following oesophagectomy in patients treated with surgery alone or multimodal therapy i.e. combined CRT before surgery. In addition, we aimed to compare mRNA expression following oesophagectomy in these patient groups to examine for differences between these strategies. We also aimed to compare the postoperative cytokine responses at protein and RNA levels for IL-10 and TNF-α i.e. to compare the results of chapters 5 and 6.

Part A   Animal Studies

Chapter 3

The establishment of a rat surgical model of reflux and consideration of strategies to reduce inflammation and tumourigenesis in this model

3.1   Introduction

Oxidative damage and inflammation from the reflux of acid and bile is central to our understanding of oesophageal injury, metaplasia and cancer (Chen and Yang, 2001). Oxidation of lipids, DNA and protein by reactive oxygen species can result in genomic instability and protein malfunction (Carr and Frei, 1999). Inflammatory processes recruit and activate inflammatory cells and directly influence cells that express their receptors (Chen and Yang, 2001).

There is a need to evaluate new approaches to inflammation in oesophageal disease, initially in experimental models. Experimental rodent models have been used to study new approaches pre-clinically (Chen et al., 1999, Chen et al., 2002b). Oesophagitis in a rodent models of oesophageal injury has been ameliorated by using the antioxidants superoxide dismutase (Wetscher et al., 1995) and DA-9601 (Oh et al., 2001). COX inhibition has been reported to reduce the incidence of adenocarcinoma in such models (Buttar et al., 2002). COX-2 inhibition in humans has come under scrutiny (Couzin, 2005). In adenoma prevention clinical trials using Celecoxib, an increase in cardiovascular events was observed, (Solomon et al., 2005, Solomon et al., 2006). Cardiovascular safety warnings have lead to a vast reduction in prescribing (Schussel and Schulz, 2006). Antioxidants have a more appealing safety profile, are an essential part of a healthy diet and are already used widely in food and drinks industries.

A rodent model of oesophageal reflux allows the study of oesophageal inflammation and its role in potential tumourigenesis at a preclinical level. Advantages of this model are that it is relatively inexpensive; the numbers of animals which can be studied are large and the experiment can be undertaken over a period of months compared to years in humans. Disadvantages of these models are that they are technically intricate and require the development of surgical, anaesthetic and postoperative care skills. Models can be associated with high mortality rates (appendix 5.4) and vigilance is needed to detect animals which are ill to avoid suffering. An additional difficulty faced in this thesis was that a rodent surgical reflux model had not been previously used in Ireland; all aspects of this had to be optimised before experimental processes could begin.

3.2   Aims

1) This first aim of chapter 3 was to establish a surgical oesophagojejunostomy model of reflux in the rodent. This model had not been studied before in Ireland; therefore careful consideration of the choice of animal to be used, the preoperative preparation and the type of anaesthesia and analgesia to be used was needed. In addition, optimisation of the surgical technique, recovering of animals postoperatively and the monitoring of the animals following surgery were of prime importance. We aimed to recognise signs of pain or complications at an early stage to prevent unnecessary suffering in ill animals

2) The second aim of chapter 3 was to find a COX-2 inhibitor and an antioxidant to potentially reduce oesophageal injury and inflammation in the oesophagojejunostomy model. We aimed determine the dosage, method of administration, and proof of absorption of these medications.

3) The overall aim of chapter 3 was to optimise conditions to enable the research in chapter 4 be performed i.e. to investigation of the degree of oesophageal inflammation in the oesophagojejunostomy model; comparing animals undergoing surgery alone and those treated following surgery with COX-2 inhibitors or vitamin C.

3.3   Optimisation of the surgical animal reflux model

3.3.1 Funding obtained to perform research

The Royal College of Surgeons in Ireland, Research Fellowship in Surgery 2003 provided funding to undertake this project and a salary was provided by Trinity College Dublin (Appendix 2).

3.3.2 Legal and ethical considerations

Ethical approval was obtained before commencement of this study from the Bioethics Committee, Trinity College Dublin, Ireland.

In accordance with the Cruelty to Animals Act 1876 (as amended by the European Communities Regulations 2002), an animal licence was obtained from the Minister for Health and Children to enable experimentation on live animals (Appendix 3). This licence detailed the number of animals and species to be used, the experimentation to be performed and anaesthesia to be administered.

The licence included Certificate A, to allow the use of anaesthesia to be dispensed with e.g. for dietary manipulation and the administration of pharmacological agents involving minimal pain. It also included Certificate B, to allow animals recover from anaesthesia i.e. following oesophagojejunostomy reflux model; for this a detailed description of the postoperative care was required.

Supervision to ensure compliance with national legislation and College policy was provided by the Bioresources Unit, Trinity College.

3.3.3 Basic animal handling education

Before the commencement of experimentation, a National Licence Training Course was undertaken in Trinity College Dublin, provided by LAST Ireland (laboratory animal science and training) (Appendix 4).

Training to gain basic proficiency in animal handling was provided by staff in the Bioresources Unit, Trinity College.

3.3.4 Choice of animal to be used in the study

Female rats were used in this reflux model, similar to several previous studies (Melo et al., 1999, Pera et al., 1989). The required number of female animals was available in-house (Bioresources unit, Trinity College); therefore costs were reduced compared to importing commercially available male animals. Female rats have a slower growth curve than males. The weight of animals was matched to previous studies, approximately 200g; therefore animals used were 25 weeks old compared to approximately 8 weeks old in studies using males. Wistar rats were used in this study; most previous animal reflux models have been performed on either Wistar rats (Kumagai et al., 2003, Melo et al., 1999, Miwa et al., 1996) or Sprague-Dawley rats (Buttar et al., 2002, Chen et al., 1999, da Costa et al., 1993, Pera et al., 1993).

3.3.5 The choice of animal reflux model to be used

The Levrat oesophagojejunostomy reflux model was chosen as the animal model of reflux. This was the first model of oesophageal reflux described in the rat (Levrat et al., 1962) and it has been one of the most widely studied animal reflux models (Buttar et al., 2002, da Costa et al., 1993, Miwa et al., 1996, Pera et al., 1989, Pera et al., 1993). In addition, a NSAID and a COX-2 inhibitor had recently been shown to provide chemoprevention of adenocarcinoma and oesophagitis in this model (Buttar et al., 2002).

3.3.6 Preoperative preparation

Animals were housed under standard conditions and a 12-hour light/dark cycle. They received commercial chow (Rat and Mouse cubes, Redmills, Ireland). Chow was withheld overnight before surgery but free access to water was maintained at all times. This was because the stomach was distended with food if surgery was performed on non-fasting animals (section 3.3.8), making surgical access more difficult. It would also result in the immediate passage of solid food past a newly created anastomosis.

All surgery was performed in a dedicated animal operating theatre used exclusively for this purpose. No other experimentation was performed in this facility at the same time. Several sets of dedicated surgical instruments were obtained to perform the surgery including microsurgical forceps, needle holders and scissors. Surgical instrumentation sets were autoclaved before use. A visual aid providing 3 x magnifications was used during surgical procedures.

3.3.7 Method of anaesthesia, provision of anaesthesia and preparation before surgery

General anaesthesia was produced by the intraperitoneal injection of premixed ketamine (90 mg/kg) and xylazine hydrochloride (3 mg/kg). This mixture was previously used for providing general anaesthesia in rats in the Bioresources Unit. In addition, subcutaneous carpofen 5 mg/kg, a NSAID analgesic (non steroidal anti-inflammatory drug) was administered following anaesthesia but before the commencement of surgery. In almost all cases, this mixture produced anaesthesia for the duration of surgery.

Oxygen was administered during the procedure via a tube placed over the rats nose with volatile anaesthesia (enflurane) added if required to maintain anaesthesia. The animal's eyes were taped closed to prevent drying out and corneal abrasion during surgery. The minimal amount of shaving necessary was performed before surgery to minimise heat loss. The animal was positioned for surgery on piece on wood, with plenty of tissue to provide insulation and protection and loose rubber bands to gently retract the legs. The operating light used was also a heat source for the animal during surgery. Topical iodine solution was applied in moderation to the abdominal skin before surgery, again to minimise heat loss. A disposable sterile adhesive surgical drape was used for all procedures, a sterile gown and gloves were worn and strict aseptic technique was employed.

3.3.8 Initial attempts at performing oesophagojejunostomy procedure and developing a postoperative care plan

Before commencing surgical procedures, The authors of previous similar animal reflux models were contacted by e-mail to seek advice (Buttar et al., 2002, Miwa et al., 1996). Helpful advice on the surgical technique and postoperative care was received from received from the Department of Pathology, Shiga University of Medical Science, Japan (Appendix 5.2).

Assistance in the optimisation of the surgical reflux procedure was provided by Mr. Narayanasamy Ravi; Lecturer in Surgery, St. James's Hospital and Department of Surgery, Trinity College and Ms. Phillippa Marks; chief technical officer, Bioresources Unit and Department of Surgery, Trinity College. It was decided to operate firstly on 12 dead rats to achieve a familiarity with the animal anatomy and to gain some initial experience with the operative technique.

To optimise the surgical technique and postoperative care, the procedure was then performed on 25 live animals. These were closely observed for post operative distress or complications; they were sacrificed during the first postoperative week and an autopsy was performed to assess the effects of the surgery. Optimisation of the postoperative care

was achieved only with the assistance of the staff of the Bioresources Unit, especially Peter Nowlan; director, Anne Brayley; chief technical officer, Brian McGlade; technical officer and Cormac O'Carroll; chief technical officer.

During the optimisation period several different approaches to performing the oesophagojejunostomy procedure were employed. The skin incision was initially quite long, almost the full length of the animal's abdomen (figure 3.1 A). The number of sutures placed to form the anastomosis and their method of placement varied (see figure 3.1 B). At the end of surgery, normal saline was initially instilled into the abdominal cavity to compensate for fluid loss during surgery. After several procedures, it was injected subcutaneously instead. Animals were recovered postoperatively in a wire bottomed cage containing 5 animals, with access to water and a heating lamp initially, which was changed to a heating pad. Postoperative analgesia was provided with subcutaneous carfofen 5mg/kg, the morning following surgery. Different strategies to reintroduce oral diet following surgery were attempted, including the provision of glucose solution, yoghurt, chocolate and chow made into a paste with water. In this initial period, it quickly became apparent when animals were ill and sacrifice was performed in these ill animals.

Figure 3.1 Initial attempt at formation of the surgical oesophagojejunostomy (3.1 A), with a magnified view of the anastomosis formed (3.1 B).
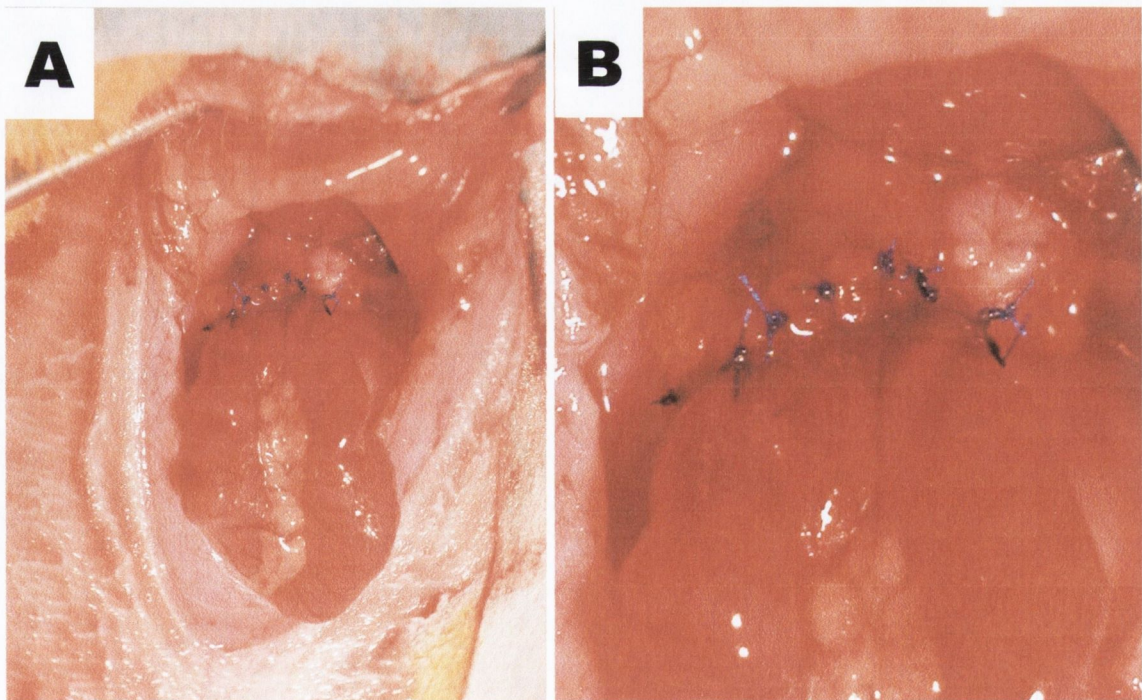
3.3.9 Initial failure of the surgical model

The first twenty operations performed on live animals produced animals that did not thrive after the surgery and sacrifice was performed on these during the first few postoperative days. Almost all of these animals demonstrated free intra-abdominal fluid at autopsy and evidence of peritonitis (figure 3.2 A). Enteric content was often found around the anastomosis when the liver was retracted (figure 3.2 B). When the anastomosis was examined in these animals there was evidence of anastomotic breakdown (figure 3.2 C + D).

The lack of success in forming a successful anastomosis led to despair and a temporary cessation in operating took place. Previous studies in the literature were carefully examined again, focusing on the operative technique and postoperative care. The Department of Pathology, Shiga University of Medical Science, Japan was contacted and informed of the difficulty in establishing the reflux model. Once again, helpful advice was received from Professor Hattori, along with an invitation to visit Japan to learn the surgical technique (Appendix 5.4).

Figure 3.2   An   Initial   autopsy   demonstrating   anastomotic   breakdown following the formation of oesophagojejunostomy. Free intra-abdominal fluid and peritonitis (3.2 A), enteric content evident on retracting the liver (3.2 B) and disruption of the anastomosis (3.2 C + D) can be seen.
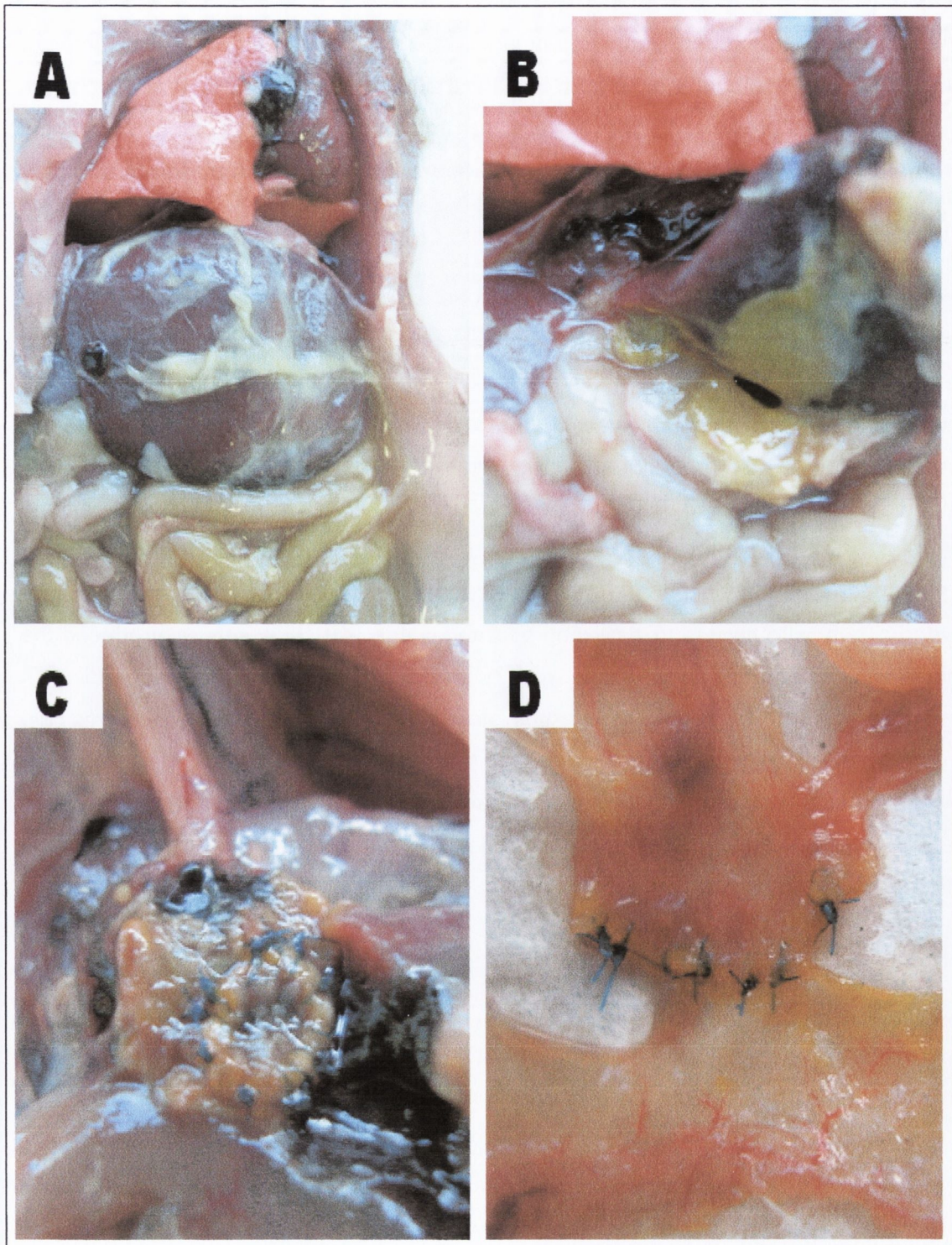
3.3.10 Optimisation of surgical technique and postoperative care

Operating was recommenced with more success. The final rats (of the initial 25) thrived after surgery and at autopsy in the first postoperative week there was no evidence of anastomotic leak. The refined oesophagojejunostomy technique was performed as follows:

A 2 cm upper midline abdominal incision was made. While protecting the vagus nerve, suture ligation of the gastro-esophageal junction was performed using 5-0 lactomer (Polysorb™, Tyco Healthcare, a gift from Healthcare 21, Dublin, Ireland). The oesophagus was divided 2 mm proximal to this ligature. An anti-mesenteric longitudinal jejunotomy was formed 4cm distal to the ligament of Treitz (figure 3.3 B). Two 7-0 polypropylene stay sutures (Surgipro™ II, Tyco Healthcare, a gift from Healthcare 21, Dublin, Ireland) were used to approximate the jejunum to the oesophagus. Three anterior wall sutures were placed and tied. The anastomosis was then turned and three posterior wall sutures were placed and tied (figure 3.3 C). The stay sutures were then tied, in total 8 sutures were placed (figure 3.3 D). The anastomosis was positioned between the lobes of the liver and the abdominal muscle layer followed by the skin were closed using 5-0 Lactomer. A diagram of the completed procedure is shown (figure 3.3 A).

Topical iodine solution was applied to the wound site following surgery. To compensate for fluid loss, 5 ml subcutaneous normal saline was administered. The rat's ears were punched to enable identification. Rats were recovered overnight, 5 per cage in wire bottomed cages, with access to a heating pad and water. Rat chow was reintroduced the morning following surgery and animals were returned to cages with standard bedding. A further dose of carpofen 5mg/kg was administered subcutaneously if any signs of pain were observed.

Following the successful formation of the rat surgical oesophagojejunostomy, therapeutic strategies to prevent inflammation and tumourigenesis in this model were then considered (Section 3.4).

Figure 3.3 Diagram and photographs of the optimised surgical oesophagojejunostomy. Diagram of oesophagojejunostomy (3.3 A), creation of jejunotomy (3.3 B), formation of anastomosis between oesophagus and jejunum (3.3 C), and completed anastomosis (3.3 D).
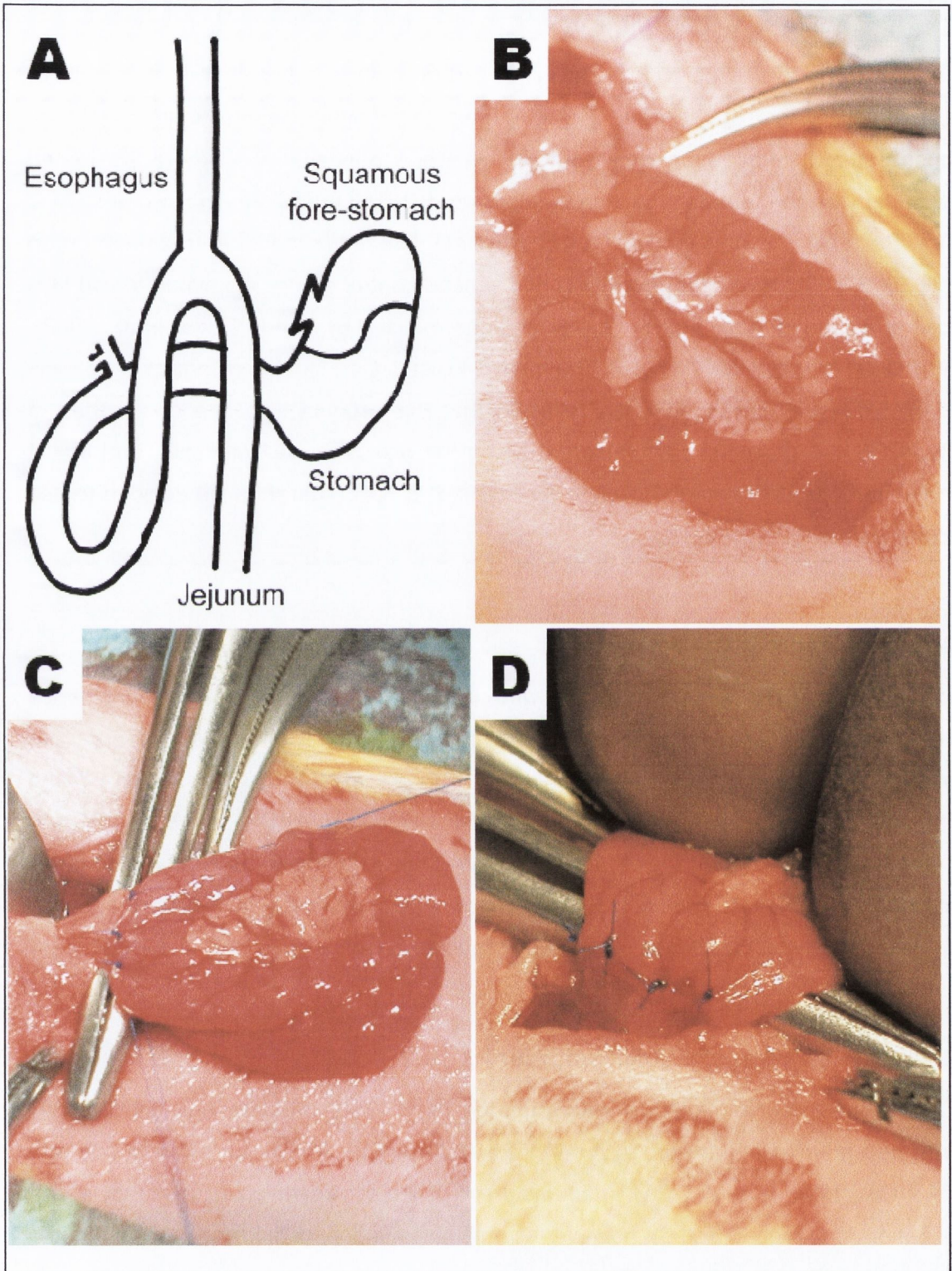
3.4 Optimisation of COX-2 inhibitor dosage and administration in the animal reflux model

3.4.1 The choice of COX-2 inhibitor

The initial idea for this research project was in part due to the publication of the results of Buttar and colleagues (Buttar et al., 2002). In their rat oesophagojejunostomy model they demonstrated reduced oesophageal adenocarcinoma and inflammation in groups treated with sulindac, a non steroidal anti-inflammatory drug (NSAID) and MF-Tricyclic, a COX-2 inhibitor. Because we were employing the same surgical reflux model, a treatment group with a COX-2 inhibitor was therefore akin to a second control group. We aimed to use MF-Tricyclic at a dose of 10mg/kg/day, the same dose as used by Buttar (Buttar et al., 2002). We attempted to obtain MF-Tricyclic from Merck Frost & Co., Quebec, Canada (appendix 6.1, 6.3). However, correspondence revealed that this product was one of a series of prototype COX-2 inhibitors manufactured during the discovery of Rofecoxib, and that it was now out of production and obsolete (appendix 6.2, 6.4). Therefore we decided to use Rofecoxib as our COX-2 inhibitor.

3.4.2 The dosage of COX-2 inhibitor

Rofecoxib doses of 0.8 and 4.1mg/kg/day were previously shown to reduce the incidence of N-methyl-N-nitrosourea (NMU) induced mammary carcinogenesis in female Sprague-Dawley rats by 40% and 42.5% after 17 weeks (Kubatka et al., 2003). Doses of 4.7 and 14.7 mg/kg/day reduced the incidence of intestinal polyposis in APC$^{\Delta 716}$ mice by 36% and 55% after 12 weeks (Oshima et al., 2001). We chose a daily dose of 4mg/kg/day Rofecoxib to administer to rats in this study. Given the approximate animal weight of 200-250g, this dose was approximately 1mg per animal per day.

3.4.3 Administration of COX-2 inhibitor

In Buttar's oesophagojejunostomy reflux model, the COX-2 inhibitor was conveniently administered to animals admixed with rat chow (Buttar et al., 2002). Correspondence with Merck & Co. (New Jersey, USA), manufacturers of Rofecoxib revealed that this was a viable option (appendix 6.5, 6.6); however scientific evaluation would take several months before this could be arranged. Therefore, rofecoxib was administered in the form of commercially available Vioxx ® syrup. This was administered as a once daily dose using a 1ml graduated syringe and feeding tip. This proved easy to administer to animals (figure 3.4)

Figure 3.4   Rofecoxib was administered to animals as a once daily dose of commercially available oral syrup
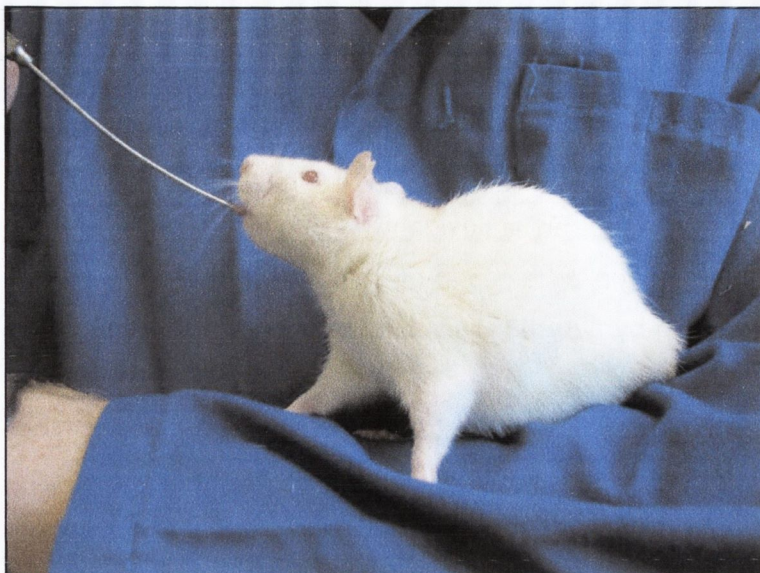
3.4.4 Monitoring of plasma COX-2 inhibitor levels

The measurement of plasma Rofecoxib levels would have required the use of technology such as high performance liquid chromatography (HPLC). This is a difficult procedure and would have taken months to set up locally. In addition, correspondence with Merck & Co. revealed that this could not normally be performed by the company for research projects (appendix 6.6). Therefore plasma rofecoxib levels were not measured and a pharmacokinetic study similar to that performed for vitamin C (section 3.4.7), was not performed.

3.5 Optimisation of vitamin C dosage and administration in the animal reflux model

3.5.1 Vitamin C (ascorbic acid) as an antioxidant

In a short term model of reflux, oesophagitis produced by duodenojejunal ligation for 24 hours was reduced by superoxide dismutase, an antioxidant (Wetscher et al., 1995). In addition, oesophagitis and NF-κB activation as a result of inserting a 3mm ring in the duodenum for 36 hours were attenuated by DA-9601, an antioxidant and NF-κB inhibitor (Oh et al., 2001).

In this study, we chose to study the antioxidant, vitamin C (ascorbic acid) at 2 doses. The reason for choosing vitamin C is that it is safe, without the side effects of NSAIDs and COX-2 inhibitors (Couzin, 2005). It is an essential part of a healthy diet (Levine et al., 1996), inexpensive to produce and is already in widespread use as a additive to prolong food life. Vitamin C is therefore an ideal candidate as a potential agent for chemoprevention of oesophageal cancer in humans.

3.5.2 The dosage and administration of vitamin C

For convenience and to ensure accurate administration, vitamin C supplementation C was administered in drinking water to animals. While administration admixed with animal chow would be more convenient, instability of vitamin C during the manufacturing process would make the concentration unreliable. A dose of 30mg/kg/day vitamin C prevented macroscopic gastric adenocarcinoma in gastrojejunostomy rats (Oliveira et al., 2003). For rats weighing 200-250g, the approximate water intake is 15mls/day. Providing a dose of 30mg/kg/day would necessitate administering 2g/l vitamin C in drinking water. We decided to provide vitamin C supplementation in tap water to two experimental groups in this study, at concentrations of 0.5g/l and 2.0g/l water. The rat chow used in this study (Rat and Mouse cubes, Redmills, Ireland) did not contain supplemental Vitamin C; this is because vitamin C is not an essential vitamin for rats; they produce their own vitamin C, unlike humans. Food grade L-Ascorbic acid (Roche Vitamins, United Kingdom) was used to make up the vitamin C solutions. Solutions were changed every second day because aqueous solutions of vitamin C degrade over time, at a rate of 11% after 7 days (OECD, 1997).

3.5.3 Pharmacokinetic study to investigate the effect of vitamin C supplementation in normal and oesophagojejunostomy rats

In chapter 4, we planned to supplement oesophagojejunostomy rats with vitamin C in an attempt to reduce inflammation and tumourigenesis. It was important to establish that supplementation at the planned doses of 0.5g/l and 2g/l would produce an increase in plasma vitamin C and antioxidant capacity levels. There is a wealth of published data on the pharmacokinetics of vitamin C supplementation in humans (Levine et al., 1996, Levine et al., 2001), and the effects of this supplementation on antioxidant levels (Carr and Frei, 1999). However, little information was found relating to vitamin C pharmacokinetics in rats, especially following gastrointestinal surgery. Therefore Vitamin C pharmacokinetics following supplementation were studied in both normal and oesophagojejunostomy rats. This was performed using 10 normal rats and 8 rats ranging from 10-22 weeks following oesophagojejunostomy. After initially receiving normal chow and tap water, Vitamin C was administered to animals at a concentration of 0.5g/l water for one week followed by 2.0g/l water for a second week. Before supplementation and at the end of the first and second week, blood was taken from animals to measure plasma vitamin C and antioxidant capacity. Sacrifice of these animals was performed at the end of the second week.

3.5.4 Measurement of plasma vitamin C levels

The plasma ascorbic acid dinitrophenylhydrazine derivative was measured using a microplate spectrophotometer method similar to that described previously (Wei et al., 1996).

Venous blood was collected from animals in the pharmacokinetic study, as described above (section 3.5.3) in lithium heparin collection tubes (Greiner bio-one Ltd., Stonehouse, Great Britain). Centrifugation was carried out immediately after collection at 3,000 revolutions per minute (RPM), for 10 minutes at 4°C. Acid deproteinisation of the supernatant (plasma) was then performed: Four parts 5% trichloroacetic acid (TCA), CCl_3COOH (made by adding 5g CCl_3COOH to 100mls double deionised water) was added to 1 part plasma while vortexing the sample. This was allowed to stand for 10 minutes at room temperature. Centrifugation was then carried out at 3,000 RPM for 5 minutes at 4°C. The supernatant was immersed in liquid nitrogen until storage at -80°C. Vitamin C analysis was performed on samples within three weeks of storage.

3.5.4.a   Reagents

Analytical quality reagents were purchased from Sigma-Aldrich Ireland Ltd., Dublin, Ireland. All reagents were freshly prepared before analysis and protected from light with aluminium foil.

3.5.4.b   Preparation of working DTC solution

DTC solution (DNPH / thiourea / copper) solution was prepared as follows. Firstly, 10 normal (10N) H_2SO_4 was prepared by adding 26.7mls 100% sulphuric acid, H_2SO_4 to double deionised water with extreme caution, making to 100mls. A 2.2% solution of 2,4-dinitrophenylhydrazine (DNPH), $C_6H_6N_4O_4$ was then made by adding 2.2g DNPH to 100mls 10N H_2SO_4. Secondly, 5% thiourea solution was prepared by adding 5g of thiourea, CH_4N_2S to 100mls water. Thirdly, 0.6% copper sulphate solution, $CuSO_4$ was made by adding 0.6g $CuSO_4$ to 100mls water. Working DTC solution was prepared by adding 5mls each of the thiourea and copper solutions to 100mls of the DNPH solution. This was protected from light.

In addition, 65% H_2SO_4 was prepared by adding 35.42mls 100% sulphuric acid, H_2SO_4 to water with extreme caution, making to 100mls.

3.5.4.c   Aqueous ascorbic acid solutions for calibration

Aqueous solutions of known ascorbic acid (vitamin C) concentration were made up as follows. Firstly, 0.176g of ascorbic acid, $C_6H_8O_6$ was added to 100mls of water to make a 100,000µmol/l ascorbic acid solution. This was diluted twice, each time adding 1 part to 9 parts water to make a 1,000µmol/l ascorbic acid solution. Dilution of 4 parts of this solution to 6 parts water produced a 400µmol/l ascorbic acid solution. Serial dilution was then performed to create solutions of 400µmol/l, 200µmol/l, 100µmol/l, 50µmol/l, 25µmol/l and 12.5µmol/l ascorbic acid. These were protected from light. Dilution of 1,000µmol/l ascorbic acid solution was also performed to obtain 500µmol/l solution.

3.5.4.d   Plasma samples

Acid deproteinised plasma samples were thawed on ice immediately prior to analysis.

3.5.4.e   Quality control- the percentage recovery of ascorbic acid from spiked plasma samples

Mixed plasma samples were spiked with a known concentration (conc) of ascorbic acid (AA) and analysis was performed to see what percentage of the added AA was recovered. This acted as a quality control step in the vitamin C analysis, by ensuring that ascorbic acid added to samples could be measured accurately.

Excess rat plasma, not used for acid deproteinisation (section 3.5.4) was mixed together, divided into cryopreserve tubes and immersed in liquid nitrogen until storage at -80°C. before analysis, the mixed plasma samples were thawed on ice.

A set of three "spiked" samples, spiking plasma with an extra 50μmol/l ascorbic acid were produced as follows. One part of 500μmol/l ascorbic acid solution was mixed with 9 parts of mixed plasma, thereby spiking plasma with an extra 50μmol/l ascorbic acid (plasma spiked with AA). One part of 500μmol/l AA solution was mixed with 9 parts of deionised water to produce a 50μmol/l AA solution (water spiked with AA). Finally, one part deionised water was added to 9 parts of the same mixed plasma (plasma spiked with water). The percentage of the spiked AA recovered during measurement was calculated: (AA conc of plasma spiked with AA) / (AA conc of water spiked with AA + AA conc of plasma spiked with water) * 100.

A second set of three "spiked" samples as described above were produced, spiking with an extra 100μmol/l ascorbic acid (one part of 1,000μmol/l ascorbic acid solution mixed with 9 parts of plasma, etc.).

3.5.4.f   Vitamin C analysis

Vitamin C analysis was performed on acid deproteinised rat plasma samples, water (reagent blank), aqueous ascorbic acid samples and the sets of three spiked samples. Four parts TCA was added to 1 part of water, the aqueous ascorbic acid solutions and quality control plasma before analysis, as above (section 3.5.4). After standing for 10 minutes and performing centrifugation at 3000RPM at 4°C for 5 minutes the supernatant was then treated the same as the thawed acid deproteinised plasma samples.

Quadruplicate 90μl volumes of acid deproteinised plasma, ascorbic acid solutions and quality control samples were added to the wells of 96 well plates. A 30μl volume of DTC solution was added to each microplate well and mixed. The 96 well plates were incubated for 3 hours at 37°C, mixing the samples every hour. After 3 hours the microplates were placed on ice and 150μl of ice cold 65% H_2SO_4 was added to each well and mixed. The microplates were allowed to return to room temperature over 30 minutes, protected from light and mixed again. The microplates were placed on a microplate reader, Multiskan plus version 2.03 (Thermo Fisher Scientific, Inc., Massachusetts, USA) and absorbance at

49

492nm (A_{492nm}) was measured, subtracting the absorbance of the reagent blank, contained in column one of each microplate.

The A_{492nm} values of aqueous ascorbic acid solutions were plotted against their known concentrations using Microsoft Excel ® software ensuring an R^2 value > 0.95. Visually, the known concentration of ascorbic acid solutions corresponded to the intensity of orange colour seen at measurement.

The ascorbic acid concentration of samples could then be calculated from their A_{492nm} using the equation of the calibration curve. The quality control samples (sets of three spiked samples) were used to show that extra ascorbic acid added to plasma samples ("spiked") could be measured ("recovered") accurately.

3.5.5 Measuring the plasma total antioxidant capacity using the ferric reducing ability of plasma (FRAP) assay

The Ferric Reducing Ability of Plasma (FRAP) was used to measure the total antioxidant capacity (Benzie and Strain, 1996). The FRAP value (expressed in µmol/l) is the ability of plasma to reduce Iron (III) present in the FRAP reagent to Iron (II).

Venous blood was collected from animals, as described above (section 3.5.3) in ethylene-diamine-tetra-acetic acid (EDTA) collection tubes (Greiner bio-one Ltd., Stonehouse, Great Britain). Centrifugation was carried out immediately after collection at 3,000 revolutions per minute (RPM), for 10 minutes at 4°C. The supernatant (plasma) was transferred to cryopreserve tube, which were immersed in liquid nitrogen. These were then placed in a freezer at -80°C until analysis was performed, within 3 weeks.

3.5.5.a Reagents

Analytical quality reagents were purchased from Sigma-Aldrich Ireland Ltd. All reagents were freshly prepared before analysis and were protected from light with aluminium foil.

3.5.5.b Preparation of working FRAP solution

Firstly, 300 mmol/litre acetate buffer solution was prepared by adding 0.31g sodium acetate trihydrate, $C_2H_3NaO_2.3H_2O$ and 1.6ml 100% acetic acid, $C_2H_4O_2$ to double deionised water. This was made up to 100mls with water. Secondly, 40 mmol/litre hydrochloric, HCl was prepared by adding 0.33mls 37% HCl to water and making to 100mls with water. Then, 10 mmol/l 2,4,6-Tri(2-pyridyl)-s-triazine, TPTZ in 40mmol/l HCl was made by adding 0.031g TPTZ to 10mls of the HCl solution. Thirdly, 20 mmol/l $FeCl_3$

solution was prepared by adding 0.54g iron (III) chloride hexahydrate, $FeCl_3.6H_2O$ to 100mls water.

Working FRAP reagent was prepared by mixing ten parts acetate buffer with one part TPTZ solution and one part $FeCl_3$ solution.

3.5.5.c   Aqueous iron (II) solutions for calibration

Aqueous solutions of known iron (II) concentration were made up as follows. Firstly, 0.278g iron (II) sulfate heptahydrate, $FeSO_4.7H_2O$ was added to 10mls water to make 100,000µmol/l Fe (II) solution. This was diluted twice, each time adding 1 part to 9 parts water to make a 1,000µmol/l Fe (II) solution. Serial dilution was then performed to make up Fe (II) solutions of 1000µmol/l, 500µmol/l, 250µmol/l, 125µmol/l, 64.5µmol/l and 32.25µmol/l.

3.5.5.d   Plasma samples

Plasma samples were thawed on ice immediately prior to analysis.

3.5.5.e   FRAP analysis

The FRAP analysis of samples was performed using 96 well plates on a microplate reader, Multiskan plus version 2.03 (Thermo Fisher Scientific). At analysis 10µl of the aqueous Fe (II) solutions or plasma samples were added to 96 well plates in quadruplicate. Freshly prepared working FRAP reagent was warmed to 37°C and added to column 1 of the microplate to act as a reagent blank. The absorbance of the FRAP reagent at 590nm was measured. A 300µl volume of FRAP reagent was added to samples and absorbance at 590nm was measured 4 minutes later. The reduction of the ferric (III) tripyridyltriazine complex to a ferrous (II) form at low pH produced an intense blue colour with a maximum absorbance at 593nm. The change in absorbance (ΔA_{590nm}) was calculated by subtracting the initial absorbance of the working FRAP reagent from the absorbance of the sample and added FRAP reagent at 4 minutes.

The ΔA_{590nm} values of aqueous Fe (II) solutions were plotted against their known concentrations using Microsoft Excel ® software ensuring an R^2 value > 0.95. The FRAP value of samples could then be calculated from their ΔA_{590nm} using the equation of the calibration curve.

3.5.6 Result of vitamin C pharmacokinetic study

3.5.6.a Calibration and Quality control for the vitamin C assay

The absorbance at 492nm (A_{492nm}) of the aqueous ascorbic acid standard solutions were plotted against their known concentrations using Microsoft Office Excel 2003 software (Microsoft Corporation, Washington, USA) (figure 3.5). The R^2 value of this calibration curve was greater than 0.99; therefore there was a close correlation between the A_{492nm} and the ascorbic acid concentration.

For every 16 rat plasma samples analysed, 2 quality control samples were analysed on the same microplate. The percentage of spiked ascorbic acid recovered from mixed plasma samples was between 95% and 100% for samples spiked with an extra 50µmol/l and between 90% and 110% for samples spiked with an extra 100µmol/l. these confirmed the reliability of the assay in measuring additional ascorbic acid spiked into plasma samples.

Figure 3.5 Calibration curve for vitamin C assay. The absorbance at 492nm (A_{492nm}) of the aqueous ascorbic acid standard solutions plotted against their known concentrations.

Vitamin C calibration curve

$y = 0.0004x + 0.0061$
$R^2 = 0.9914$
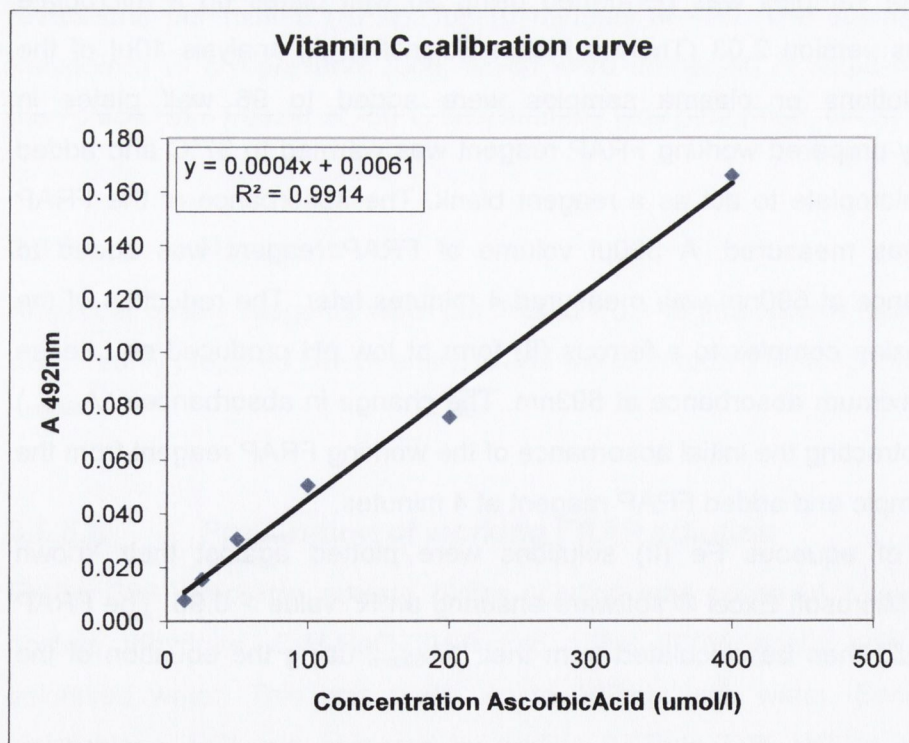
3.5.6.b Calibration for the FRAP assay

The change in absorbance at 590nm (ΔA_{590nm}) of aqueous Fe (II) solutions were plotted against their known concentrations using Microsoft Excel software (figure 3.6). The R^2 value of this calibration curve was greater than 0.99, showing a close correlation between the ΔA_{590nm} and the Fe (II) concentration.

Figure 3.6 Calibration curve for FRAP assay. The change in absorbance at 590nm (ΔA_{590nm}) of the aqueous Fe (II) solutions plotted against their known concentrations.

FRAP calibration curve- ΔA 590nm Vs FRAP

$y = 0.0006x$
$R^2 = 0.9999$
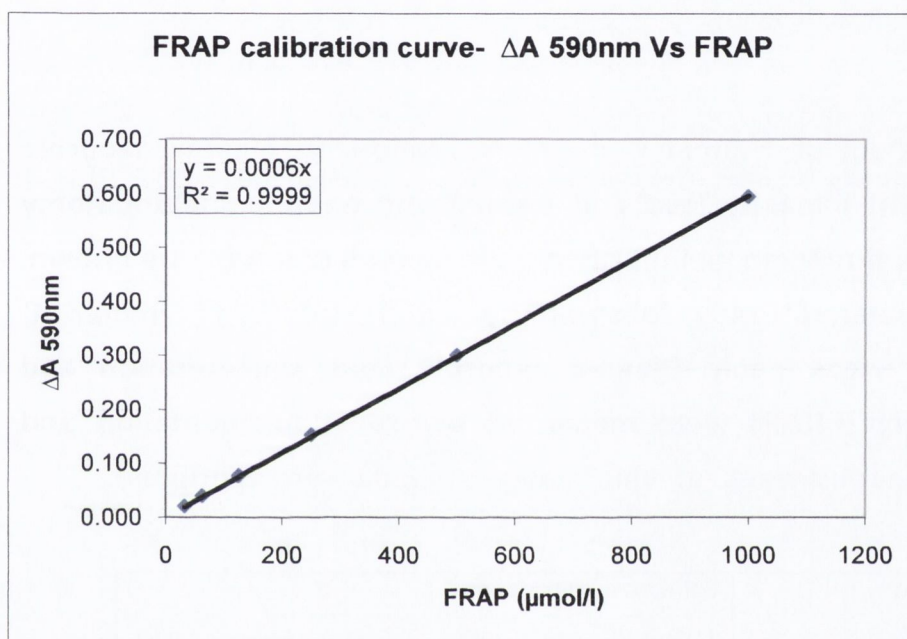
3.5.6.c Plasma Vitamin C and Antioxidant capacity levels

The results of the pharmacokinetic study are shown (table 3.1). Oesophagojejunostomy rats were found to have a significantly lower median plasma ascorbic acid level than non-operated rats before supplementation (44 µmol/l versus 70 µmol/l, p<0.01). The median plasma ascorbic acid concentration in oesophagojejunostomy rats increased significantly from 44µmol/l before supplementation to 59µmol/l after supplementation with vitamin C 0.5g/l for one week (p < 0.01); however this was still lower than the level in non-operated animals (p = 0.045). Following further supplementation with 2.0g/l vitamin C for a second week, the median plasma ascorbic acid concentration in oesophagojejunostomy rats increased from 59µmol/l to 79µmol/l (p < 0.01); this was higher than the level in non-operated animals (p < 0.01). The median plasma ascorbic acid concentration increased from 70µmol/l to 104µmol/l in non-operated rats (p < 0.01) after supplementation with vitamin C 0.5g/l for one week and remained stable at 97µmol/l following 2.0g/l supplementation for a second week.

In order to reduce phlebotomy and morbidity in nutritionally deficient oesophagojejunostomy rats, the plasma FRAP value was measured only at sacrifice. Despite this, one operated animal died following initial phlebotomy and the results in table one are based on the seven animals that survived two weeks. Oesophagojejunostomy animals that received vitamin C supplementation for two weeks achieved the same plasma antioxidant capacity as non-operated animals after the same supplementation (FRAP 298μmol/l versus 292μmol/l). The median FRAP value increased from 201μmol/l to 292μmol/l in non-operated rats supplemented with vitamin C for two weeks (p < 0.01). Therefore, in summary concentrations of 0.5g/l and 2g/l vitamin C in tap water produced a progressive and significant increase in both plasma vitamin C and antioxidant levels in rats following oesophagojejunostomy.

Table 3.1   The effect of vitamin C supplementation on plasma ascorbic acid and antioxidant capacity levels in normal and esophagojejunostomy rats. The number and median weight of animals in each category are shown. Water was administered firstly followed by 0.5g/l and 2.0g/l vitamin C solutions for one week each. Plasma ascorbic acid concentration and antioxidant capacity (FRAP) were measured before supplementation and after the first and second week, at which stage animals were sacrificed.

Normal rats (N = 10)	Solution administered		
Median weight 206g	Water	Vitamin C 0.5 g/l	Vitamin C 2.0 g/l
Average vitamin C dose (mg)	0	13	51
Median plasma ascorbic acid (μmol/l)	70	104	97
Median plasma FRAP (μmol/l)	201	258	292

Esophagojejunostomy rats (N = 7)	Solution administered		
Median weight 182g	Water	Vitamin C 0.5 g/l	Vitamin C 2.0 g/l
Average vitamin C dose (mg)	0	10	35
Median plasma ascorbic acid (μmol/l)	44	59	79
Median plasma FRAP (μmol/l)	*	*	298
Median FRAP stabilized plasma (μmol/l)	41	47	60

(* To reduce phlebotomy in esophagojejunostomy rats, plasma FRAP values were measured only at sacrifice. The FRAP value of TCA stabilised plasma, used in the measurement of ascorbic acid concentration, was determined as a surrogate.)

3.6 Discussion

In chapter 3, we succeeded in establishing a rat oesophagojejunostomy reflux model for the first time in Ireland. This involved extensive consideration and optimisation of the choice of animal, the preoperative preparation before surgery and the type of anaesthesia and pain relief used. Additionally the surgical technique, recovery of animals and postoperative care required much experimentation and re-evaluation. Initially, the surgical procedure was performed on dead animals to gain a familiarity with the anatomy and basic technical skills needed. The surgical procedures were performed in a dedicated animal research operating facility. Expert advice was attained before commencing experimentation on live animals (appendix 5). Despite this careful preparation, defects in our initial surgical technique and postoperative care became apparent. After re-evaluation, the surgical technique was successfully performed and animals thrived after the procedure.

The second part of chapter 3 involved identifying a COX-2 inhibitor and antioxidant to use in the oesophagojejunostomy model to potentially reduce oesophageal injury and inflammation. The manufacturers of a COX-2 inhibitor previously used in this animal model provided helpful advice in the choice of anti-inflammatory drug to use. We chose vitamin C as an antioxidant because of its safety, cost, availability and its already widespread use in human food products. We performed a pharmacokinetic study of vitamin C in our oesophagojejunostomy model to ensure that supplementation would lead to increased vitamin C and antioxidant levels in plasma. Based on this we chose two levels of supplementation to use in animals because progressive increases in plasma vitamin C levels and antioxidant capacity were demonstrated with these doses.

The main purpose of chapter 3 in this thesis was to establish the ground work to enable the experimentation in chapter 4. In this regard, we successfully established a rodent oesophagojejunostomy reflux model and optimised the use of a COX-2 inhibitor and an antioxidant in this model. In chapter 4, we then investigated oesophageal inflammation in this oesophagojejunostomy model, comparing animals undergoing surgery only (control animals) and those treated following surgery with COX-2 inhibitors or vitamin C.

Chapter 4

Neither antioxidants nor COX-2 inhibition protect against oesophageal inflammation in an experimental model of severe reflux

4.1 Introduction

The incidence of oesophageal adenocarcinoma has increased markedly in the western world (Devesa et al., 1998). Oxidative damage and inflammation from chronic reflux of acid and bile is central to our understanding of oesophageal injury, metaplasia and cancer (Chen and Yang, 2001). Reactive oxygen species can lead to the oxidation of lipids, DNA and protein (Carr and Frei, 1999), resulting in genomic instability and protein malfunction. Inflammation results in the production of arachadonic acid metabolites, which may recruit and activate inflammatory cells as well as directly influencing cells that express their receptors (Chen and Yang, 2001). Transcription factors are important in regulating the response to inflammation and oxidative stress, in particular the rapidly acting Nuclear Factor Kappa Beta (NF-κB) which increases transcription of genes involved in immunity, proliferation and anti-apoptosis (Karin et al., 2002). It has recently been reported that low pH and bile activates NF-κB in oesophageal cancer lines, and that a progressive increase in NF-κB activity occurs from oesophagitis, through metaplasia to adenocarcinoma in humans (Abdel-Latif et al., 2004). Recent work moreover, has shown that NF-κB pathways can be inhibited in cell lines by Vitamin C and COX-2 inhibition (Abdel-Latif et al., 2004).

These studies highlighted the need to evaluate novel anti-inflammatory approaches in oesophageal disease, initially in experimental models. Experimental rodent models have been established to study novel approaches pre-clinically (Chen et al., 1999, Chen et al., 2002b). COX inhibition has been reported to reduce the incidence of adenocarcinoma in such models: Sulindac, a non steroidal anti-inflammatory drug (NSAID) and MF-Tricyclic (a COX-2 inhibitor) reduced the incidence by 79% and 55% respectively at 28 weeks in an oesophagojejunostomy model (Buttar et al., 2002). The use of COX-2 inhibition in man has come under scrutiny (Couzin, 2005), and antioxidants may have a more appealing safety profile. In this regard, oesophagitis produced by duodenojejunal ligation for 24 hours was reduced by superoxide dismutase (Wetscher et al., 1995), and oesophagitis and NF-κB activation as a result of inserting a 3mm ring in the duodenum for 36 hours were attenuated by DA-9601, an NF-κB inhibitor (Oh et al., 2001).

In this study, the oesophagojejunostomy model, originally reported by Levrat (Levrat et al., 1962), was utilised. The antioxidant vitamin C was studied at two doses, the lower previously shown to prevent gastric cancer in a gastrojejunostomy model (Oliveira et al., 2003), along with COX-2 inhibition which has been reported to have anti-tumor effects in the Levrat model (Buttar et al., 2002). The primary focus was on inflammation rather than carcinogenesis, and so chemical carcinogens or iron overload were not incorporated into the study design.

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4.2   Aims

1) The first aim of chapter 4 was investigate the degree of oesophageal inflammation in a rodent oesophagojejunostomy model, comparing animals undergoing surgery only (control animals) and those treated following surgery with COX-2 inhibitors or vitamin C.

2) The secondary aims of chapter 4 were to compare the percentage oesophageal ulceration determined by image analysis, animal weight and animal survival between groups.

4.3   Materials and Methods

4.3.1 Legal and ethical approval

This study received ethical approval from the Bioethics Committee, Trinity College Dublin. An animal licence and relevant certificates were obtained from the Department of Health and Children, Ireland (see appendix 3). Supervision to ensure compliance with national legislation and College policy was provided by the Bioresources Unit, Trinity College.

4.3.2 Rat model of oesophageal reflux

As discussed in chapter 3, female Wistar rats weighing approximately 200g, bred in-house (Bioresources Unit, Trinity College Dublin) were housed under standard conditions and a 12-hour light/dark cycle. Rats received commercial chow (Rat and Mouse cubes, Redmills, Ireland). Chow was withheld overnight before surgery with ad libitum access to water. General anaesthesia was produced by intraperitoneal injection of premixed ketamine (90 mg/kg) and xylazine hydrochloride (3 mg/kg). Subcutaneous carpofen 5 mg/kg, a NSAID analgesic (non steroidal anti-inflammatory drug) was administered following anaesthesia but before surgery. Oxygen was administered during the procedure with enflurane added to maintain anaesthesia if required.   The oesophagojejunostomy procedure was performed as described in chapter 3. Topical iodine solution was applied to the wound site following surgery. To compensate for fluid loss, 5 ml subcutaneous normal saline was administered. The rat's ears were punched to enable identification. Rats were recovered overnight, 5 per cage, with access to a heating pad and water. Rat chow was reintroduced the morning following surgery. A further dose of carpofen was administered subcutaneously if any signs of pain were observed.

4.3.3 Experimental Design

Six weeks postoperatively, 100 surviving healthy rats were randomized into 4 groups (figure 4.1). A control group (N = 25) received normal chow and drinking water. Two groups (N = 25 each) received vitamin C dissolved in their drinking water at concentrations of 0.5g/l and 2.0g/l water, receiving on average 8mg each per day or 28mg each per day (see section 4.4.2 below). The final group (N = 25) received rofecoxib 1mg per day administered as a daily oral dose of syrup. Rats were sacrificed at 22 ± 2 weeks postoperatively.

Figure 4.1   Study design showing randomisation of 100 rats into 4 treatment groups 6 weeks after oesophagojejunostomy.
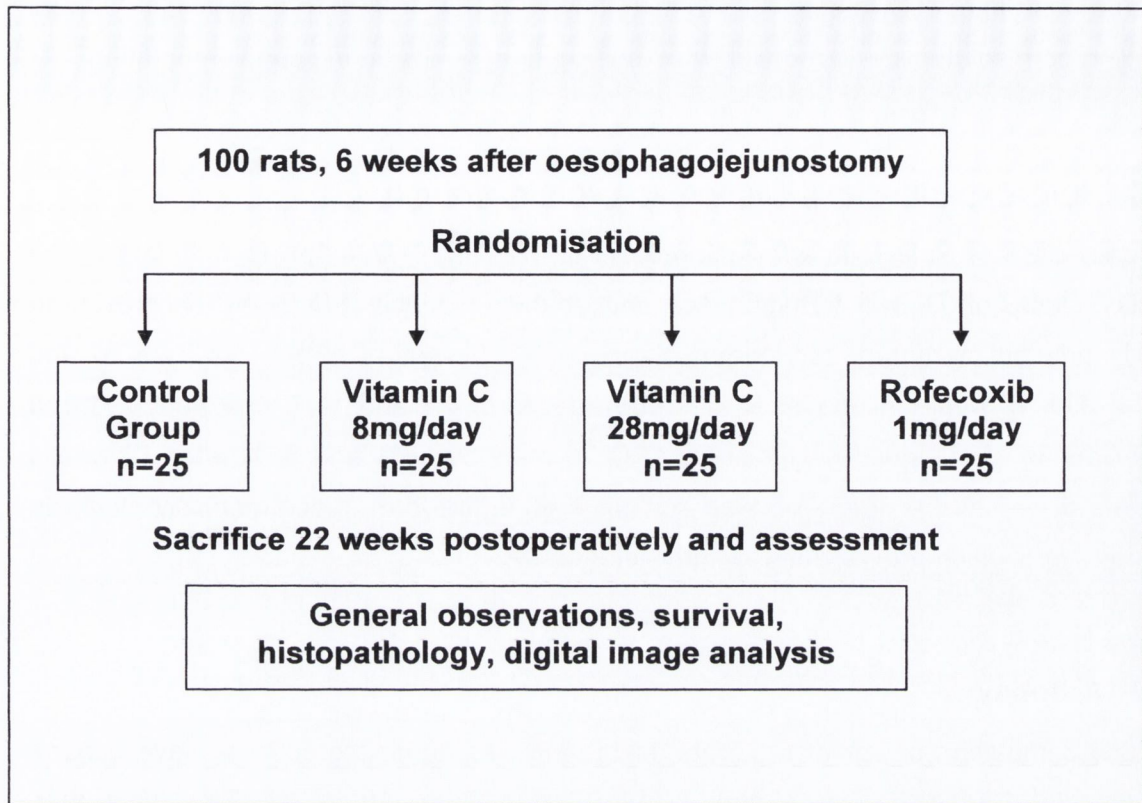
4.3.4   Sample size consideration

The number of animals or replication (r) required in each treatment group was estimated using the formula; $r = 16 (CV / d)^2$. CV, the coefficient of variation was taken as 20%, usual for biological experiments and d, the percentage difference important not to miss was considered clinically to be 20%. This formula assumes a type I error of 5% and a type II Error of 20%. This suggested that 16 animals in each treatment group would avoid missing a 20% difference between groups. We placed 25 animals in each group to allow for expected mortality.

4.3.5   Vitamin C dosage selection and administration

Vitamin C supplementation C was administered in drinking water to two of the experimental groups at concentrations of 0.5g/l and 2.0g/l water. These concentrations were based on the vitamin C pharmacokinetics in normal and operated animals which were studied in chapter 3. A dose of vitamin C similar to the lower dose prevented macroscopic gastric adenocarcinoma in gastrojejunostomy rats (Oliveira et al., 2003). The rat chow used did not contain supplemental Vitamin C. Food grade L-Ascorbic acid

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(Roche Vitamins, United Kingdom) was used to make up the vitamin C solutions. Solutions were changed every second day because aqueous solutions of vitamin C degrade over time, at a rate of 11% after 7 days (OECD, 1997). The daily intake of rats receiving both solutions was measured at multiple time points during supplementation.

4.3.6 Rofecoxib dosage selection and administration

The daily dose of Rofecoxib administered to rats was 4mg/kg/day or approximately 1mg per day. Doses of 0.8 and 4.1mg/kg/day reduced the incidence of N-methyl-N-nitrosourea (NMU) induced mammary carcinogenesis in female Sprague-Dawley rats by 40% and 42.5% after 17 weeks (Kubatka et al., 2003), doses of 4.7 and 14.7 mg/kg/day reduced the incidence of intestinal polyposis in APC$^{\Delta 716}$ mice by 36% and 55% after 12 weeks (Oshima et al., 2001). Rofecoxib was administered in the form of commercially available Vioxx ® syrup which proved easy to administer orally.

4.3.7 Autopsy

At study completion or earlier in ill rats, halothane was administered to achieve general anaesthesia. Cardiac puncture and exsanguination were performed. Rats were then placed in a CO_2 chamber until death was confirmed. A midline incision the length of the rat's body was performed. The oesophagus was removed in full from the larynx to the jejunal anastomosis, including several centimetres of afferent and efferent jejunum. The specimen was placed on card and opened along its length. Digital imaging of the oesophagus placed beside a ruler was performed. The specimen was transferred to 10% buffered formalin for fixation.

4.3.8 Specimen processing and histochemical staining

Following fixation in formalin, the oesophagus was carefully wrapped around sponge to maintain orientation during processing, which was performed in the histopathology laboratory, St. James's Hospital, Dublin. Following this, the specimen was divided into 3 longitudinal slices representing the full length and thickness of the oesophagus including the anastomosis and embedded in paraffin. Tissue sections 5μm in thickness were transferred to microscopy slides and allowed to dry.

Haematoxylin and eosin staining was then carried out using reagents purchased from Sigma-Aldrich Ireland Ltd., Dublin, Ireland. The slides were firstly immersed in two changes of xylene for 5 minutes each for dewaxing. They were then immersed in 95% ethanol for 5 minutes followed by 70% ethanol for 5 minutes and then placed in tap water

for 5 minutes. The slides were dipped into haematoxylin solution for 30 seconds and then placed into running tap water for 5 minutes. Following this, slides were dipped into eosin solution for 30 seconds and again placed into running tap water for 5 minutes. The slides were then placed into 70% ethanol for 5 minutes and xylene for 5 minutes. Cover slips were then applied to the slides.

4.3.9 Histopathological analysis

Histopathological analysis was performed by a single consultant pathologist who was blinded to experimental grouping. Where a mixed neutrophil / eosinophil inflammatory infiltrate was present, the severity of inflammation was graded as mild, moderate or severe. Severe inflammation denoted extensive ulceration, small foci of ulceration were seen in those graded moderate and no ulceration was present in mild inflammation. Mucosal squamous cell hyperplasia was scored from mild, a slight thickening, through moderate to marked, with a papillary appearance. Evidence of Barrett's oesophagus was sought in specimens, defined as intestinal type columnar metaplasia with goblet cells. Dysplasia was also looked for: abnormal cell polarity, maturation, nuclear atypia and mitotic figures, with invasion through the basement indicating carcinoma.

4.3.10   Image analysis of the oesophagus

Digital imaging software (Image J, Image Processing and Analysis in Java) was used to analyse the digital images of the opened oesophageal specimens. This software was downloaded from the National Institute of Health website (Rasband, 2005). The ruler placed beside the specimen in photographs was used to calibrate a length in pixels against its known distance in centimetres. Oesophageal dimensions were calculated. The oesophagus was traced out following conversion to greyscale and the software used to determine the oesophageal area. Using a combination of tracing and binary contrast enhancement, ulcerated oesophagus was selected and its area calculated (figure 4.2). The percentage area of oesophageal ulceration was calculated (area of ulceration / total area of oesophagus * 100) and compared between treatment groups. This was also compared against the grade of inflammation at histology.

Figure 4.2 The use of Image J, image analysis software to calculate oesophageal area and the area of ulceration. Following conversion to greyscale, tracing was used to select the oesophagus. Ulcerated oesophagus was then selected using a combination of tracing and binary contrast enhancement. The area of the oesophagus and the area of ulceration were then determined by the software.
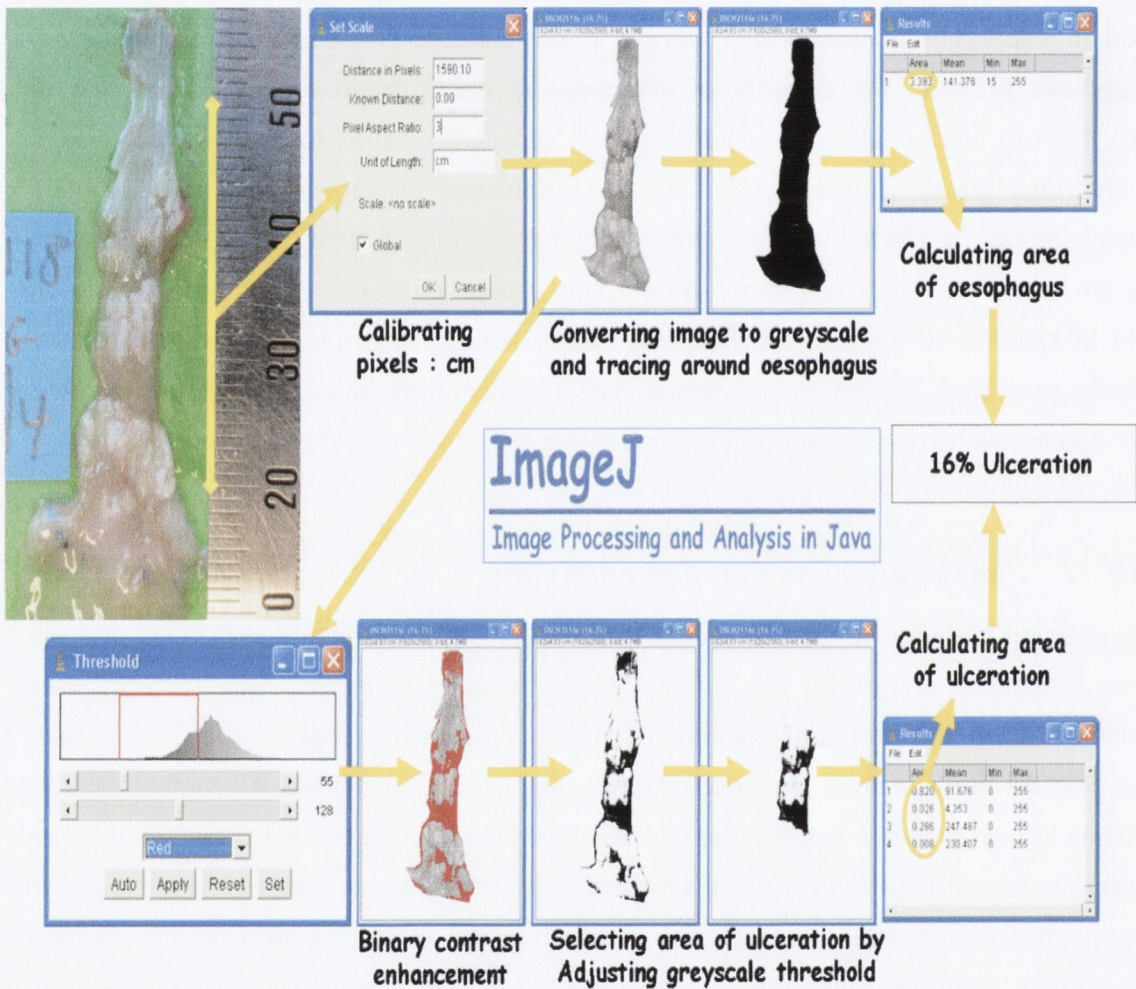
4.3.11 Statistical analysis

The primary study endpoint was the degree of oesophageal inflammation. Secondary endpoints included the percentage oesophageal ulceration determined by image analysis, animal weight and animal survival. Statistical analysis was performed using SPSS version 12.0.1 (Statistical Package for the Social Sciences, SPSS inc., Illinois, USA) and JMP IN version 4.0.4 (SAS institute inc., North Carolina, USA). Categorical data between groups was compared using the Pearson Chi-square test or Fisher's exact test. Numerical data was compared using the Wilcoxon signed ranks test and Kruskal-Wallis test. Analysis of animals completing the study as well as analysis of all study animals (Intention-to-treat) was performed. A p-value < 0.05 was considered statistically significant.

4.4   Results

4.4.1 General observations

During the study, 6 anaesthetic deaths occurred (4.9% of operations) and 17 other rats died prior to randomisation, a mortality rate of 19%. Eight unoperated rats were followed up without intervention to obtain normal oesophageal tissue.

A total of 63 out of 100 (63%) rats completed the study. Survival data demonstrated no significant difference in the survival between groups (p = 0.59). The median survival was 16 weeks in the control group, 18 weeks in those receiving vitamin C 8 mg/day, 21 weeks in those receiving vitamin C 28 mg/day and 22 weeks in those receiving rofecoxib. The reason for sacrifice or death of rats failing to reach the study endpoint was pneumonia in 14, perforated stomach in 8, malnutrition in 7, anastomotic stricture in 1 and unknown causes in 7. Analysis of the cause of death by experimental group revealed no significant differences among groups (p = 0.30).

Weight loss occurred in all animal groups, those completing the study losing a median 10% body weight after 6 weeks and 19% after 22 weeks. The weight loss was not significantly different between groups (figure 4.3) (p = 0.65).

Figure 4.3   The median weight of animals in each of the four experimental group following surgery. There were no significant differences in the weights between groups.
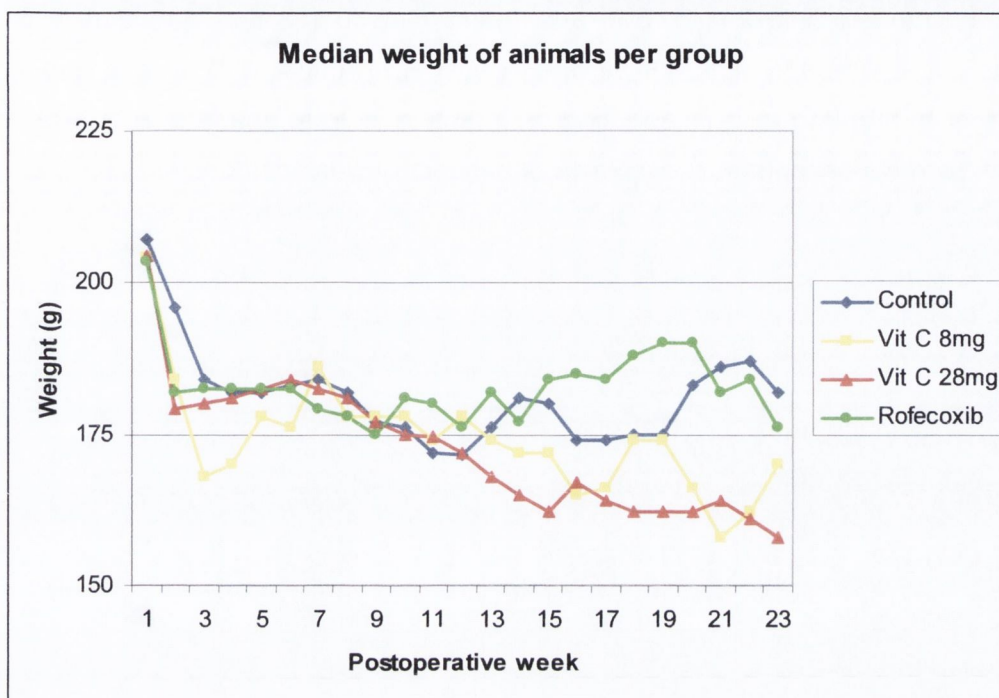
4.4.2 Dosage of vitamin C received by vitamin C groups

Animals receiving 0.5g/l vitamin C solution drank an average of 15mls per day, receiving a daily dose of 8mg of vitamin C (average of 294 rat days measured). Rats receiving 2.0g/l vitamin C drank an average of 14mls per day, corresponding to a daily dose of 28mg (average of 252 rat days measured). The effects of these doses on animal plasma concentration and antioxidant capacity are discussed in chapter 3.

4.4.3 Histopathology

A typical pathological pattern was found on the examination of oesophageal specimens (figure 4.4). Macroscopically, oesophageal ulceration beginning at the anastomosis and extended for a varying distance proximally, and areas of raised thickened epithelium were evident. Microscopically, this corresponded to oesophagitis with a mixed acute and chronic inflammatory cellular infiltrate, ulceration and areas of squamous cell hyperplasia. Oesophageal inflammation was present in all operated animals completing the study. Ulceration proved a main factor in scoring the degree of inflammation, with occasional versus extensive ulceration differentiating moderate and severe inflammation. At the study endpoint, the degree of inflammation was mild in 7 rats (11%), moderate in 29 (46%) and

severe in 27 (43%). There was no significant difference in the severity of inflammation between treatment groups at 22 weeks (table 4.1) (p = 0.17), or in all rats randomised using an intention to treat analysis (p = 0.36).

Squamous cell hyperplasia was found in all rats completing the study. This was mild in only 1 rat (1.5%), moderate in 23 (36.5%) and marked in 39 (62%). The degree of hyperplasia was not significantly different between treatment groups at 22 weeks (table 4.1), (p = 0.80), or in all rats randomised using an intention to treat analysis (p = 0.80). The presence of Barrett's oesophagus, dysplasia or carcinoma was not identified in any of our specimens.

Figure 4.4 Photographs and micrographs (haematoxylin and eosin staining, magnification x 40) of the pathological findings. (A + B) Ulceration above the anastomosis corresponding microscopically (C) to ulceration with a mixed neutrophil and eosinophil infiltration. (D + E) Areas of thickened epithelium with a cobblestone appearance corresponding microscopically (F) to squamous epithelial hyperplasia.
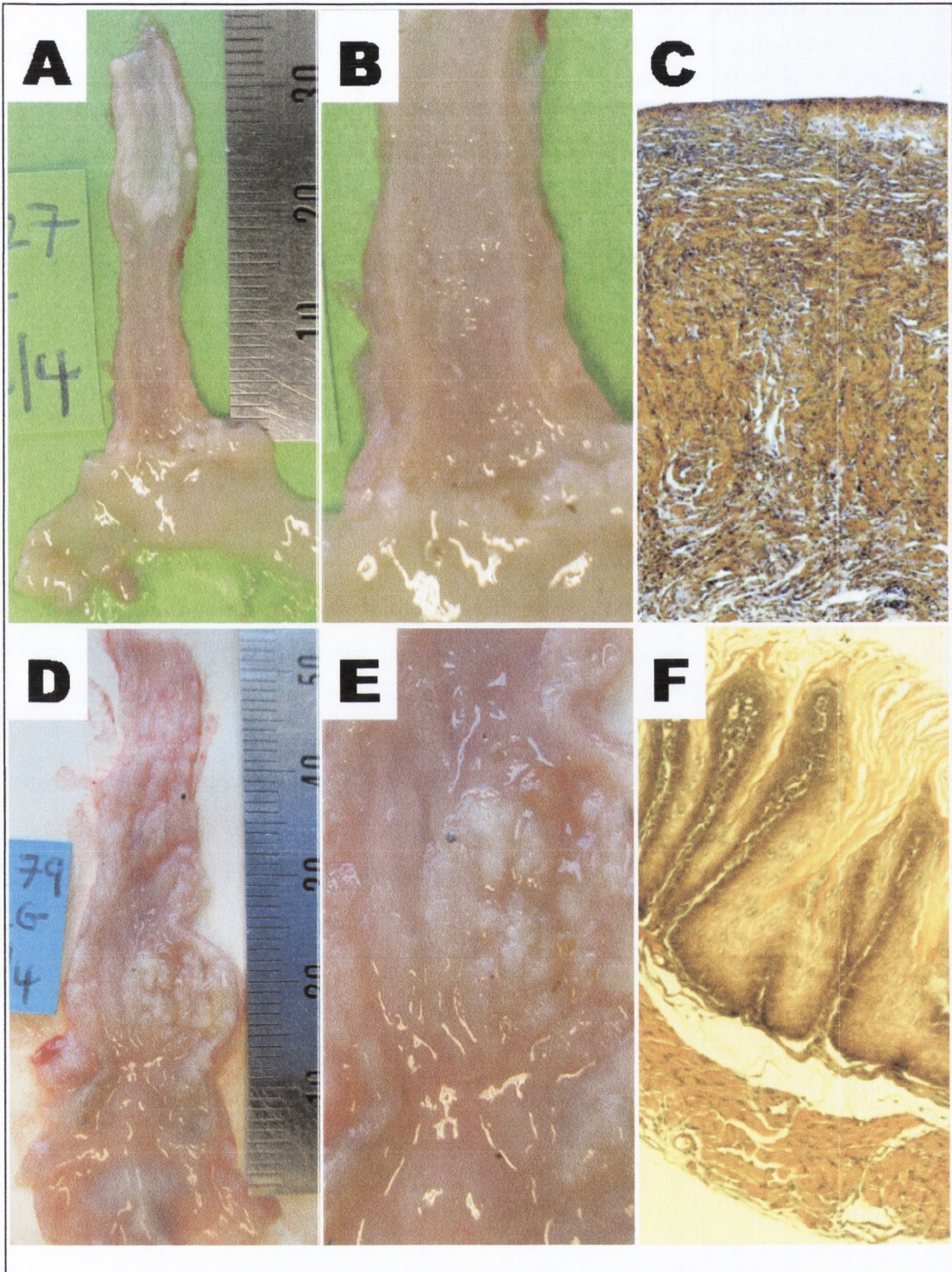
Table 4.1   Histopathological findings in animals surviving to 22 weeks postoperatively. The degree of esophageal inflammation and squamous cell hyperplasia are shown for each study group.

		Degree of inflammation		
		Mild	Moderate	Severe
Study Group	Control	2 (13%)	10 (63%)	4 (25%)
	Vitamin C 8mg	0 (0%)	7 (50%)	7 (50%)
	Vitamin C 28mg	1 (6%)	6 (38%)	9 (56%)
	Rofecoxib 1mg	4 (24%)	6 (35%)	7 (41%)
Total		7 (11%)	29 (46%)	27 (43%)

		Degree of squamous cell hyperplasia		
		Mild	Moderate	Marked
Study Group	Control	0 (0%)	5 (31%)	11 (69%)
	Vitamin C 8mg	0 (0%)	5 (36%)	9 (64%)
	Vitamin C 28mg	0 (0%)	7 (44%)	9 (56%)
	Rofecoxib 1mg	1 (6%)	6 (35%)	10 (59%)
Total		1 (2%)	23 (37%)	39 (62%)

4.4.4 Image analysis of the oesophagus

The median opened oesophageal width in operated rats at 22 weeks was 1 cm, significantly greater than the median 0.6 cm in unoperated rats ($p < 0.01$). The same was true of the oesophageal area, median 3.4 cm^2 in operated rats versus 3.05 cm^2 in unoperated rats ($p = 0.02$).

The median percentage oesophageal ulceration was not significantly different between groups (figure 4.5) ($p = 0.57$); 36% in control animals, 34% in those receiving 8 mg/day vitamin C, 49% in those treated with vitamin C 28 mg/day and 36% in the rofecoxib group.

Figure 4.5 **The percentage oesophageal ulceration found using image analysis (Image J) in each treatment group. There were no significant differences between groups**
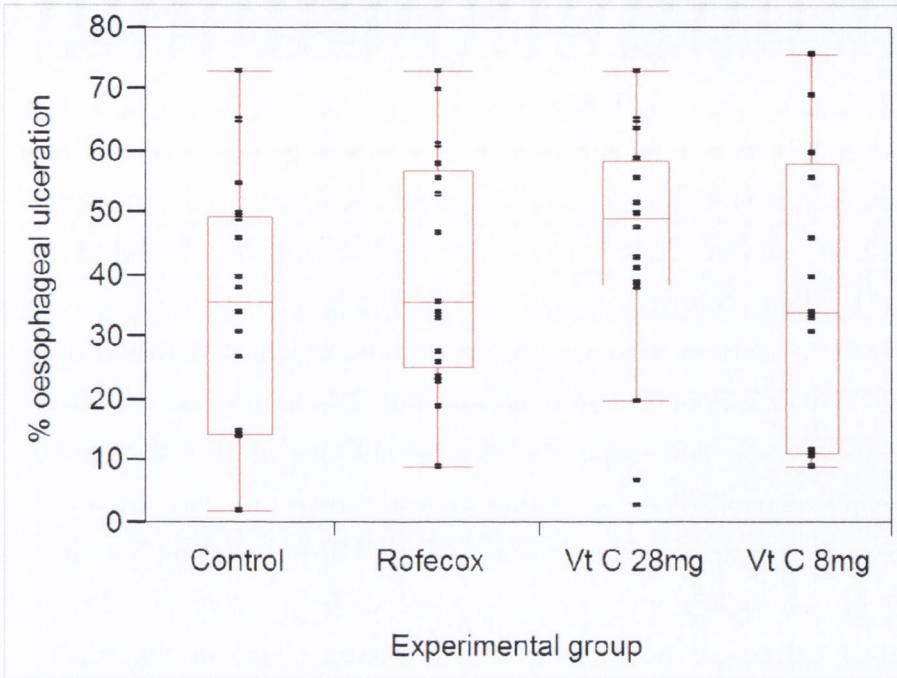
4.4.5 Degree of inflammation versus percentage ulceration

The percentage ulceration on image analysis increased significantly with the grade of inflammation at histology. The median percentage ulceration increased from 23% in mild inflammation, to 39% in moderate inflammation and 52% in severe inflammation (p < 0.01).

4.5   Discussion

In Chapter 3, a rodent model of oesophageal injury similar to that described by Levrat was established (Levrat et al., 1962). In chapter 4 animals following oesophagojejunostomy were divided into treatment groups to examine the effect of antioxidants and COX-2 inhibitors on oesophageal injury in this model. Female rats were used, matching their weight to previous studies. Female rats have a slower growth curve than males; therefore rats used were older than studies using males, median 25 weeks compared to approximately 8 weeks. The 19% mortality rate at 6 weeks in this study is the same as that reported by Buttar et al, in an oesophagojejunostomy model which randomized at 4 weeks (Buttar et al., 2002). After randomization however, just 63% completed the study compared to 91% in Buttar's study, and rats suffered a weight loss of 19% during the study compared to a weight gain of 70–80%. Weight loss and high mortality rates are often seen after oesophagojejunostomy. Miwa (Miwa et al., 1996) found an average weight increase of 14% at 50 weeks after oesophagojejunostomy; however 60% of animals in this group died before 50 weeks and the first listed cause of death was malnutrition. Similarly, Pera (Pera et al., 1993) found that only 50% of animals that underwent oesophagojejunostomy survived to their study endpoint of 32 weeks. Da Costa (da Costa et al., 1993), found that at day 14 after oesophagojejunostomy, rats had lost 22% of body weight, were anaemic (haematocrit fell by 17%) and hypoproteinaemic (fall of 14%). Chen (Chen and Yang, 2001) and Kumagai (Kumagai et al., 2003) recognized that most surgical reflux models produce lower body weight, iron nutritional status, serum albumin and fat soluble vitamin levels. Chen and colleagues (Chen et al., 2002b, Chen et al., 2000b, Chen et al., 1999) have developed an oesophagogastroduodenal anastomosis model which does not exclude stomach function allowing consistent weight gain after surgery. Kumagai (Kumagai et al., 2003) has developed this further, avoiding stomach deformity by anastomosis of a jejunal loop to the oesophago-gastric junction allowing weight gain and survival without dietary supplementation to 45 weeks.

Different causes of mortality were seen in this study compared to that of Buttar (Buttar et al., 2002); while rats in Buttar's study experienced pneumonia and anastomotic obstruction, none suffered stomach perforation, occurring in 7% of our animals and well described previously in the literature (Van Den Boogert et al., 1999).

The pathological spectrum of this study included oesophagitis, ulceration and squamous cell hyperplasia, with no incidence of Barrett's oesophagus or tumours. Some prior studies employed carcinogens, in particular nitrosamines, resulting in the production of squamous

cell carcinomas, adenocarcinomas and mixed adenosquamous carcinomas (Mirvish, 1997). Other studies used iron overload to promote oesophageal inflammation and oxidative stress (Chen et al., 1999), a high incidence of Barrett's development, ranging in length from 0.2–2 mm in 80–91% of animals, and an incidence of adenocarcinoma of 51–73% after 28-30 weeks (Buttar et al., 2002, Goldstein et al., 1997).

Barrett's oesophagus has been produced in the oesophagojejunostomy model in animal groups not receiving carcinogen or Iron treatment. Fein (Fein et al., 1998) found oesophageal columnar lining in 91% of animals 22 weeks postoperatively. Miwa (Miwa et al., 1996) found Barrett's in 100% of animals after 50 weeks. Others have had findings similar to ours; Pera (Pera et al., 1993) "despite thorough dissection" was unable to find Barrett's in oesophagojejunostomy animals after 32 weeks. Using the oesophagoduodenostomy reflux model, Barrett's was not present in some studies (Attwood et al., 1992, Oberg et al., 2000), present in low numbers in others (Ireland et al., 1996, Melo et al., 1999), and found in 86% of animals, starting as early as 10 weeks in another study (Jang et al., 2004). The fact that Barrett's was not found in our model despite careful histopathological analysis is not unusual because the presence and rate of Barrett's in animal models varies so greatly. Goldstein (Goldstein et al., 1997) recognized that the definition of Barrett's used in animal models is important and advised not to describe the presence of puckered small bowel mucosa in the oesophagus because of healing as Barrett's. Oberg (Oberg et al., 2000) suggest that columnar lining in the oesophagus could be an artefact caused by the method of embedding in paraffin and sectioning or perhaps related to the implantation of columnar epithelium by sutures. They argue that admixed squamous and columnar epithelium at the anastomosis used in some models to describe Barrett's, should be interpreted with care. The fact that female rats were used in our study was not unusual. Melo (Melo et al., 1999) used female wistar rats in their study and described Barrett's oesophagus. Pera used 50% male and 50% female animals in an oesophagojejunostomy model (Pera et al., 1989).

This study also validated an image analysis system for scoring the severity of oesophageal injury. Oesophageal ulceration can be recognised macroscopically and therefore correlating the percentage oesophageal ulceration with the degree of inflammation warranted investigation. Using Image J ® software the percentage area of oesophageal ulceration from digital images recorded at autopsy could be readily calculated. We showed a significant and progressive increase in the percentage ulceration as the degree of inflammation found at histopathological analysis increased. Thus image analysis of the percentage area of oesophageal ulceration proved a valid measurement of the severity of injury in this animal reflux model.

The principal goal of this study was to investigate the effects of the antioxidant, vitamin C and the selective COX-2 inhibitor, Rofecoxib on histopathological parameters in this model. Moderate to severe oesophagitis was found in 88% of control animals. In Buttar's study moderate to severe oesophagitis, present in 69% of control animals, was reduced significantly to 23% in the NSAID treated group and 40% in the COX-2 inhibitor group (Buttar et al., 2002). The measured Rofecoxib dose administered in this study was found to produce chemoprevention in two previous studies (Kubatka et al., 2003, Oshima et al., 2001). The Vitamin C doses were quantifiable and produced a rise in plasma vitamin C and antioxidant capacity levels in a pharmacokinetic study; this was not previously performed in an animal model of reflux. The lower dose of vitamin C used prevented macroscopic gastric adenocarcinoma in gastrojejunostomy rats (Oliveira et al., 2003). However, vitamin C or Rofecoxib treatment did not produce a reduction in the severity of oesophagitis in this animal model. This result differs from that found in Buttar's study (Buttar et al., 2002), perhaps reflecting the fact that iron overload was not used. A rat oesophagoduodenal anastomosis model with iron supplementation for 40 weeks previously found that the incidence of adenocarcinoma was not significantly reduced using high doses of the antioxidants vitamin E or selenium (Chen et al., 2000b).

Proton pump inhibitors represent the standard treatment strategy in preventing Barrett's related oesophageal cancer through acid suppression. However Sampliner (Sampliner, 1994) failed to show that high dose treatment significantly shortens the length of Barrett's in humans. The value of anti-inflammatories in preventing oesophageal cancer has recently been questioned in an extensive epidemiological study (Lindblad et al., 2005). Coupled with the recent safety concerns about COX-2 inhibitors (Couzin, 2005), there is some doubt about the usefulness of these agents in chemoprevention of intestinal cancer. Antioxidants however require further study in relation to oesophageal adenocarcinoma. Low intake of vitamins C and E has been found to correlate with the development of both types of oesophageal cancer in males (Bollschweiler et al., 2002). The fact that antioxidants are inexpensive to produce and largely without side effects makes them especially attractive as therapeutic agents.

In conclusion, this model created severe oesophageal reflux with extensive inflammation but no Barrett's or adenocarcinoma, and the systemic insult and catabolism was considerable. The study failed to demonstrate a reduction in oesophageal inflammation and ulceration using COX-2 inhibitors or two doses of the antioxidant vitamin C. It is evident that gastric exclusion should be avoided in animal models of reflux, and we would suggest that future studies with COX-2 inhibitors, antioxidants and perhaps NF-κB inhibitors should be undertaken in animal models that induce less severe inflammation.

Part B   Human Studies

Chapter 5

Analysis of the serum cytokine response to major surgery in oesophageal cancer and a comparison of patients treated with surgery alone or a multimodality regimen

5.1   INTRODUCTION

Oesophageal adenocarcinoma has increased markedly in incidence in western society (Devesa et al., 1998). The overall five year survival rate for patients with oesophageal cancer is 10% (Faivre et al., 1998). In addition, surgical treatment carries significant danger; a 14% mortality rate and a 60% risk of morbidity was described in a recent multicentre cohort study from 24 hospitals in England and Wales (McCulloch et al., 2003). A multimodality treatment approach, combining chemotherapy and radiotherapy before surgery, is increasingly used in gastrointestinal cancers such as oesophageal cancer and rectal cancer. Multimodal therapy in oesophageal cancer has been investigated extensively in a number of randomised controlled clinical trials (Nygaard et al., 1992, Le Prise et al., 1994, Apinop et al., 1994, Walsh et al., 1996, Bosset et al., 1997, Urba et al., 2001) and several meta-analyses (Fiorica et al., 2004, Malthaner et al., 2004, Urschel and Vasan, 2003). These have concluded that preoperative chemoradiotherapy (CRT) leads to pathological tumour down-staging and improved three year survival at a cost of increasing postoperative mortality (Fiorica et al., 2004, Malthaner et al., 2004, Urschel and Vasan, 2003). In our institution, a pre-operative CRT protocol (Walsh et al., 1996) is currently offered to all patients intended for curative resection of oesophageal cancer, following detailed discussion.

Major surgery results in marked metabolic and immune responses, principally mediated through the effects of hormones and cytokines. The additive effects of a thoracotomy and laparotomy during an oesophagectomy produce immense postoperative cytokine responses (Aiko et al., 2005, Fukunaga et al., 2001, Narumiya et al., 2005, Sato et al., 2002, Sato et al., 2001, Takeda et al., 2003, Tashiro et al., 1999, Tsukada et al., 2001, Yamaguchi et al., 2006, Yamauchi et al., 1998). Regulation of the systemic inflammatory response after surgery involves a complex interplay between pro-inflammatory and anti-inflammatory cytokines. The balance between these responses may influence the occurrence of complications and survival following major surgery. Differences in the pro and anti-inflammatory cytokine responses following oesophageal cancer surgery in patients treated with surgery alone or a multimodal treatment regimen have not been directly studied to date.

The cytokine response following oesophagectomy has been principally examined at a protein level, measuring individual or several cytokines in serum or plasma using Enzyme-Linked ImmunoSorbent Assay (ELISA) technology. Newer technologies have allowed more detailed investigation of the serum cytokine response following surgery. These include "biochip" array technology, able to simultaneously measuring multiple cytokines in small volumes of serum (Tu et al., 2007). This new technology invites a fresh investigation

of the serum cytokine responses following oesophagectomy. In particular it may help elucidate differences between patients treated with surgery alone or a multimodal regimen and this may help explain differences in complication rates and survival following surgery.

5.2 AIMS

1) The first aim of chapter 5 was to examine the cytokine immune response following oesophagectomy at a protein level in patients treated with surgery alone and patients treated with multimodal therapy i.e. combined CRT before surgery. We aimed to perform a detailed analysis of serum cytokine and growth factor levels using recently developed "biochip" immunoassay technology.

2) The second aim of chapter 5 was to compare the postoperative serum cytokine response following oesophagectomy in patients treated with surgery alone or a multimodal treatment regimen, to examine for differences between these strategies.

5.3   PATIENTS AND METHODS

5.3.1 Funding, patient population and study design

Funding for this project was received in the form of a Cancer Research Fellowship from St James's Hospital, Dublin 8 (Appendix 7).

Ethical approval was obtained before study commencement from the St. James's hospital and federated Dublin voluntary hospitals joint research ethics committee. Written informed consent was obtained from all patients (Appendix 8). The study was designed as a non-randomised prospective study. Consecutive patients presenting to St. James's Hospital with oesophageal cancer and treated with curative intent were included. A histological diagnosis was made in all patients using endoscopy and tissue biopsy. Disease staging included a CT (Computed Tomography) scan and fluorine-18 fluorodeoxyglucose positron emission tomography (F-18 FDG-PET). Patients with localized disease (T2-3, N0-1, M0) were discussed at multidisciplinary gastrointestinal tumour conferences; all patients in St. James's Hospital intended for curative oesophageal cancer resection are offered multimodal treatment (preoperative chemoradiotherapy before surgery) after a detailed discussion. Patients who received either surgery alone or a multimodal treatment protocol were included in this study. Those who received preoperative chemoradiotherapy were given a standard protocol of 40Gy radiotherapy with concomitant cisplatin and 5-fluorouracil as previously described (Walsh et al., 1996). The demographics and characteristics of patients were recorded including body mass index (BMI), smoking status and American society of anaesthesiologists (ASA) grade.

5.3.2 Surgical treatment, pathological assessment and postoperative course

All patients included in the study underwent either a 2-stage (abdomen and right thorax) or 3-stage (abdomen, thorax, neck) operation, with 2-field (D2 abdominal and mediastinal including sub-carinal) dissection. The operative timing, blood loss and blood transfusion requirements were documented. Patients were enterally fed from the first postoperative day via a fine bore needle catheter jejunostomy (NCJ).

Pathological examination of the resected oesophageal specimen was performed to obtain the UICC (International union against cancer) tumour stage (pT) and nodal stage (pN) (Sobin et al., 2002). The AJCC (American Joint Committee on Cancer) stage was determined (Greene et al., 2002). Tumour morphology was recorded i.e. squamous cell

carcinoma or adenocarcinoma. In patients who received preoperative CRT, Mandard's tumour regression grade was scored (Mandard et al., 1994).

The post operative course was carefully documented. The number of days spent in the high dependency unit (HDU) or intensive care unit (ICU) was recorded as well as the total postoperative hospital stay. All complications of surgery were prospectively recorded; these were classified as respiratory complications and non-respiratory complications. In case of mortality, complications, the cause death and the timing of death were noted.

5.3.3 Blood collection

In all patients, a blood sample was taken preoperatively (pre-op) and on the 1st and 7th days postoperatively (pod1 and pod7). In patients who received multimodal therapy, an additional blood sample was taken prior to commencing chemoradiotherapy (pre-CRT), where possible. Venous blood was collected from patients in Z-clot activator collection tubes (Greiner bio-one Ltd., Stonehouse, Great Britain). These were stored at room temperature for 45 minutes to allow clotting to occur. Following this, centrifugation was carried out at 3,000 revolutions per minute (RPM), for 10 minutes at 4°C.   The supernatant (serum) was transferred to cryopreserve tubes and stored at -80°C until analysis was performed.

5.3.4 Serum cytokine measurement

The evidence investigator™ cytokine and growth factor array was used to analyse serum samples (Randox laboratories Ltd., Co. Antrim, Northern Ireland, Catalogue numbers EV3513 and EV3562). This was performed using the evidence investigator™ system (figure 5.1), which simultaneously measured 12 cytokines and growth factors in 100μl of serum. Analysis was performed on specially designed 9mm x 9mm biochips, using a sandwich chemiluminescent immunoassay principle and digital imaging technology.

The 12 cytokines and growth factors measured by the array were: interleukin 2 (IL-2), interleukin 4 (IL-4), interleukin 6 (IL-6), interleukin 8 (IL-8), interleukin 10 (IL-10), vascular endothelial growth factor (VEGF), interferon gamma (IFN-γ), tumour necrosis factor alpha (TNF-α), interleukin 1 alpha (IL-1α), interleukin 1 beta (IL-1β), monocyte chemoattractant protein 1 (MCP-1) and epidermal growth factor (EGF).

A training course provided by Randox laboratories was undertaken prior to sample analysis. The analysis process involved analysis of serum samples, calibrator samples and quality control samples as described below (sections 5.3.4.a-e).

Figure 5.1   The Randox evidence investigator™ system, used to perform analysis of serum samples
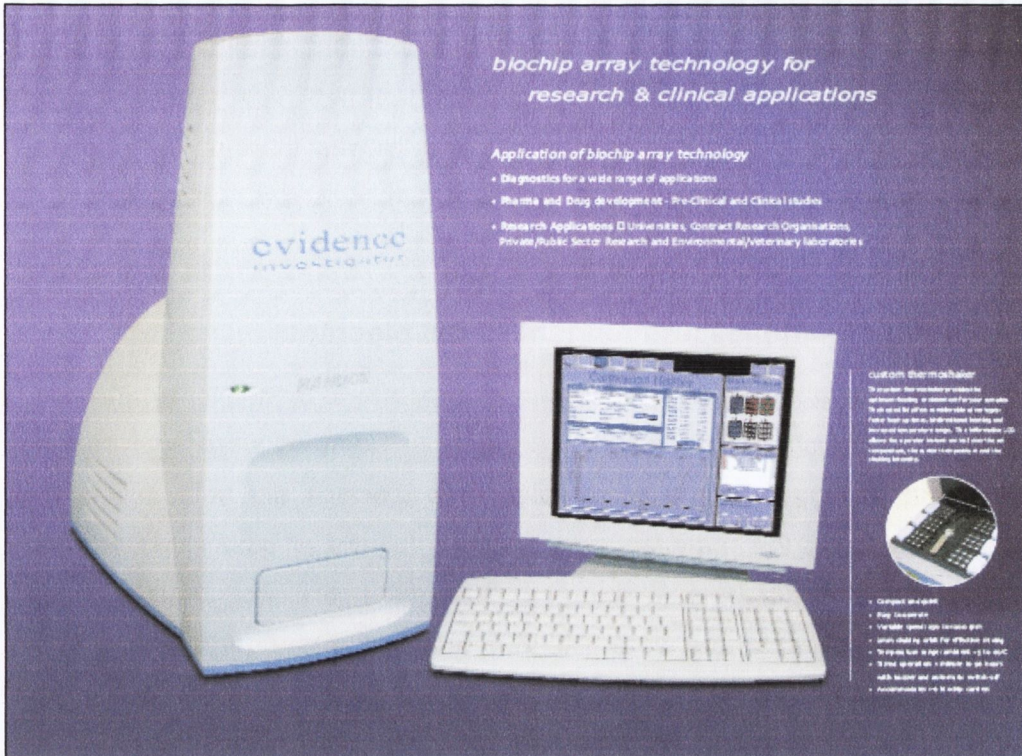
5.3.4.a   Preparation of calibrator samples

Nine cytokine and growth factor calibrator samples were provided as part of the cytokine array kit. The contents of each calibrator sample were reconstituted with 1ml of double deionised water. These were placed on a mechanical roller for 30 minutes, avoiding the formation of foam. The calibrator samples, which are stable for 4 hours were then analysed promptly (section 5.3.4.d). The results of these were used to construct a nine point calibration curve for each cytokine measured.

5.3.4.b   Preparation of quality control samples

Three quality control samples were available for use with the cytokine array kit (Randox laboratories Ltd., Catalogue number EV3562). These contained three levels of control of all the cytokines and growth factors measured by the system; low, medium and high. The contents of each control sample was reconstituted with 1 ml of double deionised water and allowed stand for 15 minutes out of bright light. The vial was then placed on a mechanical roller for 15 minutes. These control samples, which are stable for 4 hours were then analysed promptly (section 5.3.4.d). For every 8 serum samples measured, the

ninth sample measured on the same carrier was a control sample, as recommended by Randox laboratories.

5.3.4.c   Preparation of serum samples

Serum samples were removed from storage at -80°C and allowed thaw on ice prior to analysis which was performed promptly.

5.3.4.d   Addition of samples and reagent to the biochips

Before analysis all materials were equilibrated to room temperature. Biochips held in wells were placed into carriers which hold 9 wells i.e. 9 biochips. Samples were analysed in batches, ensuring that the loading time of samples onto biochips did not exceed 10 minutes, as recommended by the manufacturer. A 200μl volume of assay diluent was firstly added to each of the biochip wells, followed by 100μl of the calibrator, quality control or serum samples. The biochip carriers were placed in a handling tray and secured to a thermoshaker set at 37°C for 1 hour. The samples with diluent were then discarded, and 2 rapid wash cycles were performed using the array kit wash buffer. Four further wash cycles were then performed, allowing the wash buffer to soak the biochips for 1 minute each time. After the final wash, the wash buffer was removed from the wells by tapping onto lint free paper. Next, 300μl of the array conjugate, containing the assay specific antibodies (i.e. IL-2, IL-4, IL-6 etc.) labelled with horse radish peroxidase was added to each biochip well. The biochips were then placed on the thermoshaker for a further hour at 37°C. The reagent was then discarded and 6 wash cycles were performed as described before. Wash buffer was then added to the biochips and left until image analysis was performed within 30 minutes.

5.3.4.e   Imaging of biochips and processing of results

Each carrier of 9 biochips was processed individually for imaging, protecting carriers from light with aluminium foil. The wash buffer was first discarded from the biochip wells of each carrier. Then 250μl of working signal reagent, containing luminol and peroxide was added to each well, protecting the carrier from light. After 2 minutes, the carrier of 9 biochips was placed into the evidence investigator™ analyser. Image capture of the biochips was then performed by the system's camera and results were processed by the system software. The calibrator samples were analysed firstly to produce a nine point calibration curve for each of the 12 cytokines and growth factors. The target curve fit (r valve) of the software was 0.95; a calibration curve falling below this level is rejected and

needs to be repeated. Following this serum samples were analysed, with one biochip in each carrier used for a control sample. Using the calibration curves, the concentration of each cytokine in the serum samples was calculated by the software. The cytokine concentrations of the quality control samples were compared to their known concentration to ensure accuracy of the results.

5.3.5 Statistical Considerations

The primary endpoint of chapter 5 was to examine the overall cytokine immune response following oesophagectomy at a protein level. Preoperative (pre-op) serum cytokine and growth factor levels were compared with levels on days 1 and 7 after surgery (pod 1 and pod 7). These were performed for patients in the surgery and multimodal groups. In addition, in multimodal patients, serum cytokine levels before commencing CRT were compared to pre-op levels (after completion of CRT).

The second endpoint of chapter 5 was to compare the serum cytokine and growth factor responses following oesophagectomy in patients treated with surgery alone or a multimodal treatment regimen. Therefore serum cytokine levels were compared between the surgery and multimodal groups overall using an area under the curve (AUC) analysis, and at individual pre-op, pod 1 and pod 7 time points.

Statistical analysis was performed using SPSS version 16.0 (Statistical Package for the Social Sciences, SPSS inc., Illinois, USA). Parametric analysis was performed. Cytokine data between days for each group was compared using the paired samples T-test. Cytokine data between the surgery and multimodal groups was compared using the independent samples T-test. Categorical data between groups was compared using the Pearson Chi-square test or Fisher's exact test. A p value < 0.05 was considered statistically significant and all tests performed were two-tailed tests.

The number of patients or replication (r) required in each treatment group was estimated using the formula: $r = 16 (CV / d)^2$. CV, the coefficient of variation was taken as 20%, usual for biological experiments. A type I error of 5% and a type II error of 20% were assumed. For chapter 5, the percentage difference (d) in serum cytokine values considered important not to miss between the surgery and multimodal groups was 25%. The minimum number of patients needed in each group for the serum study was therefore 11.

5.4   RESULTS

5.4.1 Patients recruited

Thirty one patients were recruited to take part in the study, of which 14 (45%) received surgery alone (surgery group) and 17 (55%) received a multimodal treatment protocol (multimodal group). All 31 patients had serum collected preoperatively (pre-op) and on days 1 and 7 postoperatively (pod 1 and pod 7). Twelve of the 17 multimodal patients had an additional serum sample collected before the commencement of chemoradiotherapy (pre-CRT). Patients had frequently commenced chemoradiotherapy when recruited to take part in this study.

The patient characteristics, operative parameters, pathological parameters and postoperative course including the occurrence of complications and hospital stay are shown for all patients in each group (table 5.1). The postoperative complications which occurred are presented in further detail for all patients (table 5.2).

Table 5.1   Patient   characteristics,   operative   parameters,   pathological parameters and postoperative course of patients in both surgery and multimodal treatment groups

Group	Patient	Age (years)	Gender	Smoker	BMI (kg/m²)	ASA grade	T stage	N stage	Stage	Morphology	TRG	Operation	Blood loss (mls)	Transfusion (units)	Op time (hrs)	Complication	Death	HDU / ICU (days)	Hosp stay (days)
S	S1	78	M	Never	26.8	2	pT3	pN0	2a	Adeno		2 stage	2775	0	5	Yes	yes	34	34
S	S2	58	M	Current	23.5	2	pT0	pN0	0	HGD		2 stage	1400	0	7.5			4	24
S	S3	67	M	Ex	26.3	2	pT3	pN1	3	Adeno		2 stage	3000	0	4.5			2	29
S	S4	63	M	Current	17.4	2	pT3	pN1	3	Adeno		2 stage	2000	4	7	Yes		11	34
S	S5	62	M	Never	26.0	2	pT3	pN1	3	Adeno		2 stage	500	0	4			3	16
S	S6	68	F	Never	18.4	2	pT1	pN0	1	Squamous		3 stage	450	0	5.25	Yes		8	30
S	S7	71	M	Never	21.7	2	pT3	pN1	3	Adeno		2 stage	1700	0	5.5	Yes		1	48
S	S8	60	M	Never	30.7	2	pTis	pN0	0	Adeno		2 stage	355	0	6	Yes		4	23
S	S9	69	M	Current	27.3	3	pT1	pN1	1	Squamous		2 stage	2000	2	6.5	Yes		7	27
S	S10	72	F	Never	34.5	2	pT3	pN1	3	Adeno		2 stage	1700	3	5.25	Yes		4	32
S	S11	70	M	Ex	29.0	2	pT3	pN1	3	Adeno		2 stage	1700	0	6.5			4	18
S	S12	60	F	Never	27.3	2	pT1	pN0	1	Intramucosal		2 stage	600	0	5	Yes		3	27
S	S13	77	F	Never	22.9	2	pT1	pN1	1	Adeno		2 stage	400	2	4			3	17
S	S14	52	M	Current	21.6	1	pT3	pN0	2a	Squamous		3 stage	1900	0	5.25			4	22
M	M1	58	M	Ex	40.5	1	pT2	pN1	2b	Adeno	3	2 stage	2500	0	5.5	Yes		5	22
M	M2	63	M	Never	26.2	1	pT2	pN1	3	Adeno	3	2 stage	1040	0	4.5	Yes		3	27
M	M3	72	F	Never	29.1	3	pT3	pN0	2a	Adeno	3	3 stage	390	0	4	Yes		3	21
M	M4	72	M	Never	29.4	1	pT3	pN1	3	Adeno	2	2 stage	600	2	7			3	15
M	M5	57	M	Never	26.5	2	pT4	pN1	3	Squamous	4	3 stage	800	0	5			3	16
M	M6	65	M	Ex	27.1	2	pTx	pN0	2a	Adeno	1	3 stage	2200	1	6	Yes	yes	15	17
M	M7	37	M	Never	26.9	2	pT0	pN1	2b	Adeno	2	2 stage	1500	0	4			4	13
M	M8	62	M	Ex	33.3	1	pT2	pN1	2b	Adeno	3	2 stage	500	0	5			2	19
M	M9	58	F	Ex	26.8	1	pT0	pN0	0	Squamous	1	3 stage	200	2	4	Yes		4	19
M	M10	54	M	Ex	23.4	1	pT3	pN1	3	Adeno	3	2 stage	1285	0	5.5			3	20
M	M11	64	M	Ex	31.0	2	pT3	pN0	2a	Adeno	2	2 stage	1500	3	5.5			8	16
M	M12	54	M	Never	24.1	2	pT3	pN0	2a	Adeno	4	2 stage	400	0	5.5			3	16
M	M13	53	M	Current	24.3	2	pT3	pN0	2a	Adeno	4	2 stage	1700	1	6.5	Yes		1	16
M	M14	66	M	Ex	28.5	2	pT1	pN0	1	Adeno	2	3 stage	1600	1	6.5	Yes		11	48
M	M15	60	F	Never	22.8	2	pT3	pN0	2a	Squamous	2	2 stage	500	1	4.5	Yes		3	27
M	M16	70	M	Current	30.4	2	pT3	pN1	2a	Adeno	3	2 stage	3600	5	6	Yes		7	20
M	M17	37	M	Never	22.3	2	pT3	pN1	3	Adeno	4	2 stage	500	0	5.5			1	16

S = Surgery, M = Multimodal, BMI = Body Mass Index, ASA = American Society of Anesthesiologists, HGD = High Grade Dysplasia
TRG = Tumour Regression Grade, Op = Operation, HDU = High Dependency Unit, ICU = Intensive Care Unit, Hosp = Hospital

Table 5.2   Detailed description of the postoperative complications which occurred in both surgery and multimodal treatment groups

Group	Patient	Complication	Mortality (days post op)
S	S1	Pleural effusion, Pneumothorax, Myocardial infarction, Heart failure, Renal failure, Prolonged intubation	34 days
S	S2		
S	S3		
S	S4	Pleural effusion, Pneumothorax, Chylothorax	
S	S5		
S	S6	Pleural effusion	
S	S7	Pleural effusion, Anastomotic Leak (radiographic), Hydropneumothorax	
S	S8	Atelactasis, Atrial fibrillation	
S	S9	Pneumonia, Pneumothorax, Atrial fibrillation	
S	S10	Pleural effusion	
S	S11		
S	S12	Pleural effusion	
S	S13		
S	S14		
M	M1	Atelactasis	
M	M2	Deep venous thrombosis	
M	M3	Central line infection	
M	M4		
M	M5		
M	M6	Pneumonia, Prolonged intubation, Renal failure, Central line Infection	15 days
M	M7		
M	M8		
M	M9	Pleural effusion	
M	M10		
M	M11		
M	M12		
M	M13	Chylothorax	
M	M14	Pneumothorax, Tachycardia, Pulmonary embolus, Prolonged intubation	
M	M15	Pneumothorax	
M	M16	Myocardial infarction, Atrial fibrillation, Prolonged intubation	
M	M17		

S = Surgery, M = Multimodal

5.4.2 Comparability of the surgery and multimodal groups

The 14 surgery and 17 multimodal patients were compared to identify any differences between groups. The patient characteristics, operative parameters, pathologic parameters and complication rates were compared between groups (table 5.3). There was an increased age in the surgery group compared to the multimodal group (mean age: surgery group 66 years, multimodal group 59 years, p = 0.034). There were no differences found between groups when the numbers of female patients, the body mass index (BMI), smoking history (current or ex smokers) or ASA grade were examined. Almost all tumours were located in the lower oesophagus or at the oesophago-gastric junction (OGJ) with only one in each group located in the mid oesophagus. Not surprising therefore, only 6 patients (3 each in the surgery and multimodal groups) had a squamous cell carcinoma on histological examination, the rest being adenocarcinoma. In the surgery group one patient had Barrett's oesophagus with high grade dysplasia and one patient had an intramucosal carcinoma. All patients were treated with curative intent and had a laparotomy and thoracotomy performed during surgical resection; a 2 stage oesophagectomy. The number of patients who had a 3 stage oesophagectomy (an additional neck incision) was comparable in each group, as were the operating time and blood loss. No significant differences were found on pathological examination with respect to tumour stage, or the presence of lymph node metastasis.

The rate of postoperative complications was similar in both groups of patients when examined overall and when examined with the exclusion of an isolated pleural effusion. There were no significant differences when complications were analysed in terms of respiratory complications and non-respiratory complications. The length of stay in the high dependency unit or intensive care unit was not found to be different between groups. However, the postoperative length of stay in hospital was significantly greater in the surgery group than the multimodal group (mean postoperative length of stay: surgery group 27 days, multimodal group 20 days, p = 0.046).

One patient in each group died in hospital after surgery. In the surgery group this death occurred at 34 days after surgery, complicated by a pleural effusion, pneumothorax, myocardial infarction, heart failure, renal failure and prolonged intubation. In the Multimodal group a death occurred 15 days post-op following pneumonia, a central line Infection, renal failure and prolonged intubation.

Table 5.3   Comparison of the surgery and multimodal groups in terms of patient characteristics, operative parameters, tumour pathology and complication rates

	Surgery (n = 14)	Multimodal (n = 17)	p
Patient and operative parameters			
Mean age (yrs) (Std. Error Mean)	66.2 (2.0)	58.9 (2.5)	0.034
Gender			
Male	10	14	0.671
Female	4	3	
Smoker			
Current or ex smoker	6	9	0.713
No	8	8	
Mean BMI (kg/m^2) (Std. Error Mean)	25.3 (1.3)	27.8 (1.1)	0.133
ASA grade			
I - II	13	16	1.000
III - IV	1	1	
Mean operation time (hrs) (Std. Error Mean)	5.6 (0.4)	5.3 (0.3)	0.498
Mean blood loss (mls) (Std. Error Mean)	1445 (253)	1236 236	0.553
Mean blood transfusion (units) (Std. Error Mean)	0.9 0.4	1 (0.4)	0.772
Operation type			
2 stage	12	12	0.412
3 stage	2	5	
Mean HDU / ICU stay (days) (Std. Error Mean)	6.6 (2.2)	4.1 (0.6)	0.258
Mean post-op hospital stay (days) (Std. Error Mean)	27.1 (2.6)	20.5 (2.0)	0.046
Pathological analysis			
Tumour histology			
Squamous cell	3	3	1.000
Adeno	9	14	
HGD / Intramucosal	2	0	
Pathologic tumour (T) stage			
T0 - T2	6	6	0.667
T3 - T4	8	11	
Pathologic nodal (N) stage			
N0	8	9	0.815
N1	6	8	
Tumour Regression Grade (TRG) (Multimodal group)			
TRG 1- 2		6	
TRG 3		7	
TRG 4- 5		4	
Complications			
No Complication	6	8	0.815
Complication	8	9	
Complication excluding isolated pleural effusion	5	8	0.524
Respiratory complication	8	7	0.376
Non respiratory complication	4	5	1.000
Mortality	1	1	1.000

BMI = Body Mass Index, ASA = American Society of Anesthesiologists
HDU = High Dependency Unit, ICU = Intensive Care Unit, HGD = High Grade Dysplasia

5.4.3 Calibration and quality control results

Nine point calibration curves for each of the 12 cytokines and growth factors were generated by the evidence investigator software as described (section 5.3.4.e). The target curve fit (r valve) of the software for each curve was 0.95. An r value between 0.98 and 1.00 was achieved for all of the 12 cytokines and growth factors (table 5.4). Therefore a valid calibration was performed. In addition every ninth sample analysed was a quality control sample and all quality control samples fell within the acceptable limits set by Randox laboratories.

Table 5.4   Calibration report obtained using Evidence Investigator™ software; the target curve fit (r value) was achieved for all of the 12 calibration curves

Evidence Investigator Calibration Report

Panel:	Cytokine
Date of Calibration:	Wednesday, May 25, 2005
Time of Calibration:	11:25 AM

Calibrator Batch No.:	0758
Calibrator Expiry Date:	Friday, September 30, 2005
Operator:	seamuse

Analyte	Calibration Range	Target curve fit (r)	Curve fit (r)
IL2	0.00-1000.00pg/ml	0.95	0.98
IL4	0.00-1000.00pg/ml	0.95	1.00
IL6	0.00-350.00pg/ml	0.95	1.00
IL8	0.00-1600.00pg/ml	0.95	1.00
IL10	0.00-600.00pg/ml	0.95	1.00
VEGF	0.00-2000.00pg/ml	0.85	1.00
IFNG	0.00-1000.00pg/ml	0.95	1.00
TNFA	0.00-1000.00pg/ml	0.95	1.00
IL1A	0.00-500.00pg/ml	0.95	1.00
IL1B	0.00-500.00pg/ml	0.95	1.00
MCP1	0.00-1200.00pg/ml	0.95	1.00
EGF	0.00-500.00pg/ml	0.95	1.00

5.4.4 Serum results

The overall serum cytokine and growth factor results for surgery and multimodal patients are shown (table 5.5). The mean (and standard error of the mean in parenthesis) serum levels expressed in pg/ml, pre-op and on pod 1 and pod 7 are shown for the surgery (14 patients) and multimodal (17 patients) groups. Pre-CRT levels are also shown for 12 of the 17 multimodal patients. Significant differences in cytokine levels on pod 1 and pod 7 compared to pre-op levels are indicated. These serum results are also displayed graphically (figure 5.2).

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Table 5.5 **Mean and (standard error of mean) serum cytokine and growth factor levels for the surgery and multimodal groups, pre-op and on pod 1 and pod 7. Significant differences in cytokine levels on pod 1 and pod 7 compared to pre-op levels are indicated.**

Surgery Group

Cytokine	Unit	pre-op (n = 14)		pod 1 (n = 14)		pod 7 (n = 14)	
IL-2	pg/ml	38	(18)	24	(10)	31	(18)
IL-4	pg/ml	3	(2)	3	(1)	4	(2)
IL-6	pg/ml	55	(25)	258 ‡	(30)	109 †	(35)
IL-8	pg/ml	21	(5)	45 ‡	(6)	34	(6)
IL-10	pg/ml	4	(4)	4	(2)	2	(2)
VEGF	pg/ml	155	(34)	140	(25)	415 ‡	(86)
IFN-γ	pg/ml	4	(2)	3	(1)	3	(1)
TNFα	pg/ml	7	(2)	7	(2)	9	(1)
IL-1α	pg/ml	3	(1)	3	(1)	3	(1)
IL-1β	pg/ml	4	(3)	2	(2)	2	(2)
MCP1	pg/ml	503	(75)	641	(95)	643 †	(86)
EGF	pg/ml	140	(31)	85 †	(21)	120	(31)

† $p < 0.05$ versus pre-op   ‡ $p < 0.01$ versus pre-op
No differences between Surgery and Multimodal groups

Multimodal Group

Cytokine	Unit	pre-CRT (n = 12)		pre-op (n = 17)		pod 1 (n = 17)		pod 7 (n = 17)	
IL-2	pg/ml	6	(3)	8	(3)	7	(3)	8	(3)
IL-4	pg/ml	2	(1)	1	(1)	2	(0)	1	(0)
IL-6	pg/ml	8	(2)	15	(7)	268 ‡	(20)	54 †	(16)
IL-8	pg/ml	22	(4)	15	(3)	45 ‡	(8)	33 ‡	(6)
IL-10	pg/ml	0	(0)	0	(0)	2	(1)	1	(1)
VEGF	pg/ml	202	(42)	144	(24)	163	(35)	340 †	(101)
IFN-γ	pg/ml	3	(2)	5	(4)	1	(0)	2	(1)
TNFα	pg/ml	7	(1)	5	(1)	6	(1)	6	(1)
IL-1α	pg/ml	2	(1)	1	(0)	2	(1)	2	(1)
IL-1β	pg/ml	5	(4)	1	(0)	1	(0)	1	(0)
MCP1	pg/ml	429	(45)	436	(37)	647 ‡	(73)	567 †	(73)
EGF	pg/ml	129	(27)	128	(19)	94 †	(16)	98	(21)

† $p < 0.05$ versus pre-op   ‡ $p < 0.01$ versus pre-op
No differences between Surgery and Multimodal groups

5.4.5 Perioperative changes in serum cytokine levels in surgery and multimodal patients

The perioperative cytokine and growth factor levels were analysed for both the surgery and multimodal groups, comparing the levels on pod 1 and pod 7 with the pre-op levels. Significant changes after surgery were found in both groups of patients, for 5 of the 12 cytokines measured (table 5.5 and figure 5.2).

Serum IL-6 levels increased significantly after surgery on pod 1 compared to pre-op levels in both surgery and multimodal groups. Despite a fall in IL-6 levels on pod 7, they remained significantly elevated compared to pre-op levels in both groups.

Serum IL-8 levels were also significantly raised on pod 1 compared to pre-op levels in both groups. Levels of IL-8 fell on pod 7 but remained significantly higher than pre-op levels in the multimodal group.

Serum VEGF levels on pod 1 were not significantly different from pre-op levels; however levels increased by pod 7 when they were significantly higher than pre-op in both the surgery and multimodal groups.

Serum MCP1 levels were significantly greater on pod 7 compared to pre-op in both groups; in the multimodal group this increase was also significant on pod 1.

In both surgery and multimodal groups, serum EGF levels fell significantly on pod 1. By pod 7, EGF levels had returned towards those seen pre-op.

For 7 of the 12 cytokines measured (IL-2, IL-4, IL-10, IFN-γ, TNF-α, IL-1α and IL-1β), no significant change in serum levels were detected on pod 1 or pod 7 compared to pre-op levels in either the surgery or multimodal groups (table 5.5 and figure 5.2).

Twelve of the 17 multimodal patients had blood taken before commencement of chemoradiotherapy (pre-CRT). There were no statistically significant differences in the levels of the 12 cytokines and growth factors before commencing chemoradiotherapy (pre-CRT) when compared to the levels preoperatively (pre-op).

Figure 5.3   (Following six pages)

Perioperative serum cytokine levels for the surgery group (clear boxes) and multimodal group (shaded boxes). The graphs display the mean and standard error of the mean. The pre-op levels are compared with those on pod 1 and pod 7 (and pre-CRT) and differences are shown (‡ p < 0.01, † p < 0.05).

Figure 5.3 (Continued)

Serum IL-2

S Vs MM, p = 0.118

Group
- Surgery
- Multimodal

Group
- Surgery
- Multimodal

Error Bars: +/- 1 SE

Serum IL-4

S Vs MM, p = 0.698

Group
- Surgery
- Multimodal

Group
- Surgery
- Multimodal

Error Bars: +/- 1 SE

Figure 5.3 (Continued)

Serum IL-6

S Vs MM, p = 0.393

Group
■ Surgery
□ Multimodal

Group
I Surgery
⋮ Multimodal

Error Bars: +/- 1 SE

Serum IL-8

S Vs MM, p = 0.726

Group
■ Surgery
□ Multimodal

Group
I Surgery
⋮ Multimodal

Error Bars: +/- 1 SE

Figure 5.3 (Continued)

Serum IL-10

S Vs MM, p = 0.339

Group
■ Surgery
□ Multimodal

Group
I Surgery
I Multimodal

Error Bars: +/- 1 SE

Serum VEGF

S Vs MM, p = 0.794

Group
■ Surgery
□ Multimodal

Group
I Surgery
I Multimodal

Error Bars: +/- 1 SE

Figure 5.3 (Continued)

Serum IFN-γ

Serum TNF-α

Figure 5.3 (Continued)

Serum IL-1α

Group
- ■ Surgery
- □ Multimodal

Group
- I Surgery
- I Multimodal

Error Bars: +/- 1 SE

Serum IL-1β

Group
- ■ Surgery
- □ Multimodal

Group
- I Surgery
- I Multimodal

Error Bars: +/- 1 SE

Figure 5.3 (Continued)

Serum MCP-1

S Vs MM, p = 0.659

Group
- Surgery
- Multimodal

Group
- Surgery
- Multimodal

Error Bars: +/- 1 SE

Serum EGF

S Vs MM, p = 0.648

Group
- Surgery
- Multimodal

Group
- Surgery
- Multimodal

Error Bars: +/- 1 SE

5.4.6 Comparison of the cytokine response following oesophagectomy in surgery and multimodal patients

Cytokine and growth factor levels were compared between the surgery and multimodal groups.

Across all 12 serum cytokine and growth factor responses, no statistically significant differences were found between the groups using an area under the curve (AUC) analysis (p values shown in figure 5.2). In addition, no intergroup differences were evident when cytokine and growth factor values were compared at pre-op, pod 1 or pod 7 time points for all 12 cytokines and growth factors analysed (table 5.5 and figure 5.2).

5.4.7 The effect of postoperative complications on the serum cytokine responses

In designing this study, we did not aim to compare the serum cytokine responses in patients with or without complications following oesophagectomy; because we were already comparing surgery and multimodal treatment groups, our study was underpowered to examine this. We were unsurprised to find no significant differences in the serum cytokine responses in patients with or without complications, in the surgery or multimodal groups.

5.5 Discussion

The primary endpoint of chapter 5 was to examine the serum cytokine immune response following oesophagectomy. This was performed for patients treated with surgery alone and for patients treated with multimodal therapy i.e. combined CRT before surgery. The cytokine response following oesophagectomy has been principally examined at a protein level. This study examines this more extensively than previously possible using recently developed array technology.

The second endpoint of chapter 5 was to compare the postoperative serum cytokine response following oesophagectomy in patients treated with surgery alone or a multimodal treatment regime. This chapter compares the cytokine response at protein level for 14 surgery and 17 multimodal patients. Immunosuppression following combined CRT has been described in oesophageal cancer patients; however the control group consisted of healthy volunteers, not oesophageal cancer patients treated with surgery alone (Heidecke et al., 2002). Damaging immunological effects of preoperative CRT have been reported following surgery for advanced rectal cancer in a similar number of patients to this study (Wichmann et al., 2003). This is the first study comparing cytokine response following oesophagectomy in patients treated with surgery alone or multimodal therapy.

"Biochip" array technology was used to measure the cytokine response at a protein level; this allowed simultaneous measurement of 12 cytokines. These were measured for 105 serum samples and numerous calibrator samples and control samples; in total, more than 1,500 immunoassay reactions were performed.

The patient characteristics, operative and pathological parameters of the surgery and multimodal groups in chapter 5 were compared; the only statistically significant difference detected was that the mean age of the multimodal patients was less than that of the surgery patients. Analysis revealed no intergroup differences in serum cytokine levels between surgery and multimodal patients. Additionally, serum cytokine analysis of all patients, comparing those greater or less than the mean age revealed no differences. Similar serum cytokine results were found following laparotomy or laparoscopy where postoperative cytokine levels were unaffected by patient age or BMI (Torres et al., 2007). The postoperative course was similar with regard to complications and the number of days spent in the high dependency or intensive care unit. The postoperative inpatient stay was longer in the surgery group. The multimodal patients in this study were all admitted to

hospital for preoperative CRT; this probably allowed for easier hospital discharge planning following surgery.

Significant changes in the levels of 5 serum cytokines were found in both the surgery and multimodal groups after oesophagectomy. In summary, we found a significant rise in serum IL-6 and IL-8 levels on pod 1, a significant rise in serum VEGF and MCP1 by pod 7 and a significant fall in serum EGF levels on pod 1 compared to pre-op levels. For the other 7 cytokines measured (IL-2, IL-4, IL-10, IFN-γ, TNF-α, IL-1α and IL-1β), we found no significant change in the postoperative levels in either group.

We found a significant increase in pro-inflammatory serum IL-6 levels on the first postoperative day (pod 1) following oesophagectomy in both surgery and multimodal patients. Similar findings were described postoperatively following oesophagectomy (Yamauchi et al., 1998, Tashiro et al., 1999, Sato et al., 2002, Takeda et al., 2003, Aiko et al., 2005, Narumiya et al., 2005, Fukunaga et al., 2001, Shibasaki et al., 2006), abdominal aortic aneurysm repair (Berguer et al., 1999, Bown et al., 2003), gastrectomy (Tashiro et al., 1999), pulmonary lobectomy (Yamauchi et al., 1998) and liver resection (Kimura et al., 2006). The magnitude of the IL-6 response following surgery correlates with the extent of surgery; levels following conventional oesophagectomy were significantly greater than those following thorascopic oesophagectomy (Fukunaga et al., 2001), oesophagectomy using a "mini-thoracotomy" (Narumiya et al., 2005), gastrectomy (Tashiro et al., 1999), cholecystectomy (Yamauchi et al., 1998, Yamaguchi et al., 2006) or mastectomy (Yamauchi et al., 1998). In our study, we found no significant difference in the serum IL-6 response following oesophagectomy in patients treated with surgery or multimodal therapy. A reduced serum IL-6 response was described following rectal cancer surgery in patients treated with preoperative CRT (Wichmann et al., 2003). In contrast, an enhanced IL-6 response was reported following lung cancer resection in patients who received preoperative chemotherapy (Endo et al., 2004). Differences between these studies and ours may reflect differences in the magnitude of an oesophagectomy compared to e.g. an anterior resection. On the first postoperative day following anterior resection, mean serum IL-6 levels were found to be 227pg/ml in the surgery group and 141pg/ml in the multimodal group (Wichmann et al., 2003). Following oesophagectomy in our study corresponding mean IL-6 levels were 258pg/ml in the surgery group and 268pg/ml in the multimodal group.

We found a significant increase in pro-inflammatory serum IL-8 levels on pod 1 in both study groups following oesophagectomy. Similar findings have been described following major surgery e.g. oesophagectomy (Berguer et al., 2000, Fukunaga et al., 2001, Sato et al., 2001, Tsukada et al., 2001, Sato et al., 2002, Takeda et al., 2003, Aiko et al., 2005,

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Kimura et al., 2006). An increased IL-8 response correlates with the extent of surgery; levels were greater following transthoracic oesophagectomy than following thorascopic oesophagectomy (Fukunaga et al., 2001). Following oesophagectomy Il-8 levels have correlated with the occurrence of pulmonary complications (Tsukada et al., 2001); in addition concentrations in bronchoalveolar lavage fluid were 20 times greater than that in peripheral blood on pod 1 (Sato et al., 2001). In this study, we found no difference in the postoperative serum IL-8 response in patients treated with surgery or multimodal therapy. Postoperative Il-8 levels following neoadjuvant CRT have not been examined in previous studies.

This study showed increased serum VEGF levels following oesophagectomy, reaching statistical significance on pod 7 in surgery and multimodal patients. VEGF levels were previously reported to be significantly raised on pod 5 and pod 10 following oesophagectomy (Spence et al., 2002). The authors found that although VEGF may arise from wound healing or be derived from tumour cells, activated platelets which produce VEGF were also increased postoperatively. We demonstrated a significant reduction in VEGF levels in multimodal patients preoperatively (following CRT) compared to before CRT. A previous study demonstrated no change in serum VEGF levels during the course of preoperative CRT for oesophageal cancer (McDonnell et al., 2001). This study, again in contrast to our results, found that serum VEGF levels in multimodal patients fell on pod 1 following oesophagectomy, returning to pre-op levels on pod 5 (McDonnell et al., 2001). We demonstrated no difference in preoperative serum VEGF levels or the postoperative response in patients treated with surgery or multimodal therapy. These groups have not been compared in previous studies; the prior mentioned study compared the post oesophagectomy VEGF response in multimodal patients to the response in patients undergoing 6 varying procedures for non-malignant disease (McDonnell et al., 2001).

We found that serum MCP1 levels were increased significantly in the multimodal group on pod 1 and in both groups on pod 7. Increased MCP1 levels were previously shown to occur in the first 24 hours after major surgery, remaining raised for several days afterwards (Shibasaki et al., 2006, Kimura et al., 2006). The postoperative serum MCP1 response correlates with the severity of surgery and was greater following oesophagectomy than following gastric or colorectal surgery (Shibasaki et al., 2006). Plasma MCP1 levels were higher in patients with organ dysfunction following liver resection (Kimura et al., 2006). This study demonstrated no significant differences in serum MCP1 levels between surgery or multimodal patients.

In this study, serum EGF levels were shown to fall significantly for patients in both study groups on pod 1 following oesophagectomy. Serum EGF levels have been studied after liver resection for metastasis (de Jong et al., 2004) and using the same biochip array technology following cataract surgery (Tu et al., 2007); in both studies, no post-op change

in serum EGF levels was uncovered; however aqueous humour EGF levels increased in patients after cataract surgery (Tu et al., 2007). We found no difference in the postoperative serum EGF response in surgery and multimodal patients.

There were no changes in serum IL-10, TNF-α, IL-2, IL-4, IFN-γ, IL-1α and IL-1β levels following oesophagectomy in this study.

We found no significant postoperative change in anti-inflammatory IL-10 levels following oesophagectomy in surgery or multimodal patient groups. Increases in serum or plasma IL-10 following surgery were previously found following oesophagectomy (Sato et al., 2002, Sato et al., 2001, Yamaguchi et al., 2006, Yamauchi et al., 1998), abdominal aortic aneurysm repair (Bown et al., 2003) and liver resection (Kimura et al., 2006). Plasma IL-10 levels were found to correlate with the extent of surgery; increases were found following oesophagectomy and pulmonary lobectomy, but not following mastectomy or laparoscopic cholecystectomy (Yamauchi et al., 1998). Increased plasma IL-10 levels have been associated with increased morbidity; following liver resection they correlated with postoperative infections (Kimura et al., 2006) and in severely injured patients detectable IL-10 was associated with the development of sepsis (Sherry et al., 1996). Differences observed between the above studies and ours are difficult to explain. In our serum study, measured serum IL-10 levels were consistently low e.g. the mean levels on the first postoperative day were 4pg/ml in the surgery group and 2pg/ml in the multimodal group. An understanding of the changes in IL-10 levels following surgery was felt to be important, and these were further studied in chapter 6 using PCR technology.

We did not demonstrate any postoperative change in pro-inflammatory serum TNF-α levels in the surgery or multimodal groups. For multimodal patients, there was a significant reduction in serum TNF-α preoperatively (following CRT) compared to before CRT, however numerically the median reduction was small (from 8 pg/ml pre-CRT to 6 pg/ml pre-op). Plasma TNF-α levels were previously undetectable after oesophagectomy, gastrectomy, mastectomy and laparoscopic cholecystectomy (Yamauchi et al., 1998) and levels remained unchanged following abdominal aortic aneurysm repair (Bown et al., 2003). In patients treated with surgery alone for rectal cancer, serum TNF-α levels increased on pod 5 (Wichmann et al., 2003). In patients treated with preoperative CRT, serum TNF-α levels previously remained unchanged during CRT for oesophageal cancer (Zemanova et al., 2005), and were unchanged after CRT and after surgery for rectal cancer (Wichmann et al., 2003). We found no difference in the postoperative serum TNF-α response in patients treated with surgery or multimodal treatment protocols. Along with IL-10, Changes in TNF-α levels following surgery were further studied in chapter 6 using PCR technology.

Relatively few studies have examined serum or plasma IL-2, IL-4 or IFN-γ levels following surgery. The plasma levels of IL-4 following liver resection have been examined and no change was found in the postoperative period (Kimura et al., 2006). Small but significant changes in serum IFN-γ levels were detected 18 hours following cataract surgery but no change in IL-2 or IL-4 levels were observed (Tu et al., 2007). These three cytokines have been investigated extensively postoperatively in isolated blood mononuclear cells after stimulation. The intracellular production of IL-2 and IFN-γ in helper T-cells was found to be reduced on the 2nd day after abdominal aortic aneurysm repair (Berguer et al., 1999). The same authors examined the change in T-cell intracellular IL-4 production following surgery and found no change following aortic aneurysm surgery or carotid endarterectomy (Berguer et al., 1999). The same was true following laparoscopic cholecystectomy (Berguer et al., 2000). Isolated blood mononuclear cells have been isolated and stimulated with staphylococcal enterotoxin B (SEB) in patients undergoing major upper gastrointestinal surgery, aortic aneurysm repair or in patients diagnosed with acute pancreatitis. IL-2 levels were found to increase significantly 1 week after major upper gastrointestinal surgery (Sweeney et al., 2005), and following open abdominal aortic aneurysm repair (Sweeney et al., 2002), but no change in levels were detected in patients diagnosed with acute pancreatitis (Sweeney et al., 2003). There was no change in IFN-γ levels detected following aneurysm repair (Sweeney et al., 2005). In patients undergoing transhiatal or transthoracic oesophagectomy, the levels of IL-2, IL-4 and IFN-γ, as well as IL-10, IL-12 and IL-13 produced by stimulated blood cells were found to be significantly lower 24 hours postoperatively in both patient groups (van Sandick et al., 2003).

To summarise the published data for IL-2, IL-4 and IFN-γ, no changes in serum or plasma levels have been shown following major surgery, although various changes have been detected in the levels produced by stimulated monocytes. This is consistent with the findings of the present study. In this study, no differences in levels were detected between surgery or multimodal patient groups.

No change was demonstrated in IL-1α levels post-op in this study. This is poorly studied in the literature, however the same has been found following cataract surgery (Tu et al., 2007). Serum or plasma IL-1β levels were previously found to be undetectable following oesophagectomy, gastrectomy etc. (Yamauchi et al., 1998), and no significant change was demonstrated following aneurysm repair (Bown et al., 2003). These studies are consistent with the present study where no change in the IL-1β level was found. When the post-oesophagectomy responses of these two cytokines were compared in surgery and multimodal patients, no differences were found.

In summary, chapter 5 demonstrates a complex postoperative interplay between pro-inflammatory and anti-inflammatory serum cytokines in patients treated with surgery or multimodal therapy for oesophageal cancer. No differences were detected when surgery

and multimodal treatment groups were directly compared. Factors responsible for an observed difference in post-operative mortality and overall survival between patients treated with surgery alone or multimodal therapy are still unknown (Fiorica et al., 2004, Malthaner et al., 2004, Urschel and Vasan, 2003). In chapter 6, we examine in detail the perioperative mRNA expression of pro-inflammatory TNF-α and anti-inflammatory IL-10 in oesophageal cancer patients treated with surgery alone or multimodal therapy

Chapter 6

Analysis of pro-inflammatory and anti-inflammatory cytokine mRNA expression following oesophageal cancer surgery in patients treated with surgery alone or a multimodal treatment regimen

6.1 INTRODUCTION

As discussed in chapter 5, the surgical treatment of oesophageal cancer is associated with significant mortality and morbidity (McCulloch et al., 2003). The role of multimodal therapy in oesophageal cancer has been studied extensively in randomised controlled clinical trials (Nygaard et al., 1992, Le Prise et al., 1994, Apinop et al., 1994, Walsh et al., 1996, Bosset et al., 1997, Urba et al., 2001). The findings of 3 meta-analyses have concluded that preoperative chemoradiotherapy (CRT) produces pathological tumour down-staging and improved three year survival but increases postoperative mortality (Fiorica et al., 2004, Malthaner et al., 2004, Urschel and Vasan, 2003).

The complex interaction between pro-inflammatory and anti-inflammatory cytokines may influence the occurrence of complications and survival following major surgery. The marked postoperative cytokine responses following oesophagectomy have been studied mainly at a protein level (Aiko et al., 2005, Fukunaga et al., 2001, Narumiya et al., 2005, Sato et al., 2002, Sato et al., 2001, Takeda et al., 2003, Tashiro et al., 1999, Tsukada et al., 2001, Yamaguchi et al., 2006, Yamauchi et al., 1998). In Chapter 5, recently developed "biochip" array technology was used to examine the cytokine and growth response following oesophagectomy in patients treated with surgery alone or a multimodal treatment regimen. When 12 cytokines were examined at 3 time-points between surgery and multimodal patients, no differences between the two groups were detected, overall or at individual time-points.

Most studies of the cytokine responses following major surgery have utilised protein technology i.e. detecting the level of the cytokine in plasma or serum. A recent expansion in genomic technology and knowledge has made possible the study of the postoperative cytokine response at an RNA level. Real time quantitative polymerase chain reaction (RQ PCR) is one such technology, uniquely capable of amplifying and detecting low levels of cytokine messenger ribonucleic acid (mRNA) expression following surgery (Duggan et al., 2006, Huang et al., 2007, Zimmermann et al., 2003). Along with recently developed protein technology, studied in chapter 5, RQ PCR has the potential to uncover differences in the postoperative response between patients treated with surgery alone or multimodal therapy. This may uncover clues to the observed differences in complication rates and survival in these two groups of patients.

Pro-inflammatory tumour necrosis factor alpha (TNF-α) and anti-inflammatory interleukin 10 (IL-10) have been described as the "prototypic proinflammatory and anti-inflammatory cytokines" (Duggan et al., 2006). Because of a high affinity for cell surface receptors, the detection of protein levels in serum may be difficult. Levels of TNF-α in particular have proven difficult to measure in serum following major surgery (Bown et al., 2003, Yamauchi et al., 1998). These are two ideal cytokine targets to study at an RNA level following major

surgery e.g. oesophagectomy. This has been performed successfully in patients following cardiac surgery with cardiopulmonary bypass (Duggan et al., 2006).

6.2 AIMS

1) The first aim of chapter 6 was to examine the cytokine immune response following oesophagectomy, comparing this at an RNA level in patients treated with surgery alone and patients treated with multimodal therapy i.e. combined CRT before surgery. We aimed to perform a detailed examination of the mRNA expression of two principle cytokines, IL-10 and TNF-α in RNA isolated from whole blood and peripheral blood mononuclear cells (PBMCs).

2) The second aim of chapter 6 was to compare postoperative IL-10 and TNF-α mRNA expression following oesophagectomy in patients treated with surgery alone or a multimodal treatment regimen, to examine for differences between these strategies. We also aimed to compare the results of chapters 5 and 6, i.e. to compare the postoperative cytokine responses at protein and RNA levels for IL-10 and TNF-α.

6.3 Patients and Methods

6.3.1 Patient population and study design

As in chapter 5, ethical approval was obtained before study commencement from the St. James's hospital and federated Dublin voluntary hospitals joint research ethics committee. Written informed consent was obtained from all patients.

The study was designed as a non-randomised prospective study. Consecutive patients presenting to St. James's Hospital with oesophageal cancer and treated with curative intent were included. All the patients included in the PCR studies in chapter 6 also had serum analysed in chapter 5. A histological diagnosis was made in all patients using endoscopy and tissue biopsy. Disease staging included a CT (Computed Tomography) scan and fluorine-18 fluorodeoxyglucose positron emission tomography (F-18 FDG-PET). Patients with localized disease (T2-3, N0-1, M0) were discussed at multidisciplinary gastrointestinal tumour conferences.

As discussed in chapter 5, all patients in St. James's Hospital intended for curative oesophageal cancer resection are offered multimodal treatment (preoperative chemoradiotherapy before surgery) after a detailed discussion. Patients who received either surgery alone or a multimodal treatment protocol were included in this study. Those who received preoperative chemoradiotherapy were given a standard protocol of 40Gy radiotherapy with concomitant cisplatin and 5-fluorouracil as previously described (Walsh et al., 1996).

The demographics and characteristics of patients were recorded including body mass index (BMI), smoking status and American society of anaesthesiologists (ASA) grade.

6.3.2 Surgical treatment, pathological assessment and postoperative course

All patients included in the study underwent either a 2-stage (abdomen and right thorax) or 3-stage (abdomen, thorax, neck) operation, with 2-field (D2 abdominal and mediastinal including sub-carinal) dissection. The operative timing, blood loss and blood transfusion requirements were documented. Patients were enterally fed from the first postoperative day via a fine bore needle catheter jejunostomy (NCJ).

Pathological examination of the resected oesophageal specimen was performed to obtain the UICC (International union against cancer) tumour stage (pT) and nodal stage (pN)

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(Sobin et al., 2002). The AJCC (American Joint Committee on Cancer) stage was determined (Greene et al., 2002). Tumour morphology was recorded i.e. squamous cell carcinoma or adenocarcinoma. In patients who received preoperative CRT, Mandard's tumour regression grade was scored (Mandard et al., 1994).

The post operative course was carefully documented. The number of days spent in the high dependency unit (HDU) or intensive care unit (ICU) was recorded as well as the total postoperative hospital stay. All complications of surgery were recorded prospectively; these were classified as respiratory complications and non-respiratory complications. In case of mortality, complications, the cause death and the timing of death were noted.

6.3.3 Blood collection

Similar to chapter 5, in all patients, a blood sample was taken preoperatively (pre-op) and on the 1st and 7th days postoperatively (pod1 and pod7). Venous blood was collected in Ethylene diamine tetracetic acid (EDTA) collection tubes (Greiner bio-one Ltd., Stonehouse, Great Britain) and transported directly to the laboratory, where the extraction of total ribonucleic acid (RNA) from whole blood and peripheral blood mononuclear cells (PBMCs) was commenced within 15 minutes.

6.3.4 Extraction of total ribonucleic acid (RNA) form whole blood

6.3.4.a   Extraction of total RNA form whole blood using the QIAamp® RNA blood mini kit

In all patients except two (one in each group, section 6.3.4.b), RNA was isolated from whole blood using the QIAamp® RNA blood mini kit (Qiagen Ltd, West Sussex, United Kingdom, Catalogue number 52304). RNA in blood is contained in leucocytes; in this method of extracting RNA from blood, lysis of the erythrocytes is performed followed by RNA extraction from the remaining leucocytes. The following is a description of the protocol followed for samples; this is similar to the manufacturer's protocol (Qiagen, 2006a) with some minor adjustments following discussion with Qiagen technical support.

The extraction of RNA from whole blood was commenced within 15 minutes of collecting venous blood in EDTA collection tubes. A 1.5ml volume of blood was mixed with 7.5mls of buffer EL (erythrocyte lysis buffer, a QIAamp kit component) in a 15ml polypropylene tube. This was incubated on ice for 15 minutes, mixing twice by vortexing during the incubation.

Centrifugation was performed at 500 revolutions per minute (RPM) for 10 minutes at 4°C after which the supernatant was completely removed and discarded. The cell pellet at the bottom of the polypropylene tube was resuspended with 3mls of buffer EL and vortexed. The centrifugation step was repeated, again completely removing and discarding the supernatant.

The cell pellet contained in the polypropylene tube was made up of leucocytes. This contained both mononuclear cells and polymorphonuclear cells. In the same patients these were also selectively isolated as described below (section 6.3.6.a). Although not part of the QIAamp kit protocol, in several cases, the viability of the cell pellet was tested. This was performed by resuspending the pellet in 1ml ice cold phosphate buffered saline. A 10µl volume of the homogenous cell suspension was added to 20µl ethidium bromide / acridine orange solution and viewed under fluorescent microscopy as described below (6.3.5.b). This confirmed a high percentage (>95%) viability of leucocytes in the cell pellet.

The first step of ribonucleic acid (RNA) extraction was commenced by adding 600µl buffer RLT with added β-Mercaptoethanol (QIAamp kit component) to the cell pellet while stirring and mixing with the transfer pipette. These leukocyte samples were then stored at -80°C and RNA extraction was completed when a batch of 10 samples had been stored.

To complete RNA extraction, the leukocyte samples with added buffer RLT were thawed on ice. Each sample was pipetted onto a QIAshredder homogenisation column and centrifugation was carried out at maximum speed (17,000 RPM) at 4°C for 3 minutes. This produced a homogenised cell lysate which was collected in the 2ml collection tube of the QIAshredder column.

A 600µl volume (or volume equal to the lysate) of 70% ethanol was added to the cell lysates and mixed by pipetting. Up to 700µl of this mixture was added to a QIAamp spin column and this was centrifuged at 10,000 RPM for 15s at 4°C. The flow through into the collection tube was discarded. This step was repeated with the remaining cell lysate and ethanol mixture.

The optional on-column DNase digestion step was performed using RNase free DNase (Qiagen Ltd, Catalogue number 79254). Firstly, 350µl of wash buffer RW1 was pipetted onto the QIAamp spin column. Centrifugation was performed; 10,000 RPM for 15s at 4°C, discarding the flow through. The DNase working solution was formed by gently mixing 10µl DNase stock solution with 70µ buffer RDD. An 80µl volume of DNase working solution was pipetted directly onto the silica-gel membrane of the QIAamp spin column and placed at room temperature for 15 minutes. Finally, wash buffer RW1 was used to wash the QIAamp spin column as described before.

The QIAamp spin column was transferred to a new collection tube. A 500µl volume of buffer RPE (with added ethanol) was added to the column and left for 1 minute. Centrifugation was carried out at 10,000 RPM for 15s at 4°C, discarding the flow through.

The column was washed for a second time with 500µl buffer RPE which was again left for 1 minute. Centrifugation at 10,000 RPM at 4°C was performed, this time for 2 minutes, again discarding the flow through. To ensure any residual ethanol was removed from the QIAamp spin column, this was then placed in a new collection tube and centrifugation was performed at 17,000 RPM at 4°C for one minute. This collection tube was then discarded.

To elute RNA from the QIAamp spin column, this was transferred to a 1.5ml cryopreserve collection tube. A 30µl volume of RNase-free water was pipetted onto the silica-gel membrane of the column and left for 3 minutes. The RNA was eluted by centrifugation at 10,000 RPM for 1 minute at 4°C. To obtain a higher concentration of RNA, this eluate was once again placed onto the membrane of the QIAamp spin column and left again for 3 minutes. Elution was then performed by centrifugation as before. The RNA samples were immediately placed on ice, a 1µl aliquot was set aside to assess the quantity and purity of extracted RNA, and the RNA sample was stored at -80°C.

6.3.4.b   Extraction of total RNA form whole blood using QIAzol and the QIAamp® RNA blood mini kit.

Two initial patients had RNA extracted from whole blood using QIAzol® lysis reagent (Qiagen Ltd, West Sussex, United Kingdom, Catalogue number 79306). The initial step involved in the homogenisation of blood using Qiazol was very quick; however, the rest of the RNA extraction process was found to be more cumbersome and RNA yields were lower. Therefore RNA extraction using the QIAamp kit (section 6.3.4.a) was used for all remaining patients.

QIAzol® lysis reagent performs lysis of all components of blood including erythrocytes and leukocytes; then this mixture can be stored for future RNA extraction. The following is a brief description of the protocol followed, similar to a standard protocol (Molecular_Research_Centre, 2007).

The extraction of RNA from whole blood was commenced within 15 minutes of collecting venous blood in EDTA collection tubes. A 1.25ml volume of blood was mixed with 3.75mls of QIAzol® and 100µl of 5N acetic acid in a 15ml polypropylene tube (5 N acetic acid was prepared by mixing 1ml >99% acetic acid with 2.48mls RNase-free water). The cap was placed on the tube and the contents were mixed well by vortexing and allowed to stand at room temperature for 5 minutes. At this stage, the samples could be stored at -80°C and RNA extraction completed in batches.

For completion of RNA extraction, the samples were thawed on ice. When thawed, 1ml of chloroform was added to each tube which was closed and shaken vigorously for 15 seconds. This was placed at room temperature for 5 minutes and then centrifugation was performed at 15,000 RPM for 15 minutes at 4°C. This separated the mixture into 3

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phases; the upper phase was the aqueous phase containing the RNA. The aqueous phase was transferred to a fresh tube and 2.5mls of isopropanol was added to precipitate the RNA. The samples were placed at room temperature for 10 minutes and then centrifugation was performed at 15,000 RPM for 8 minutes at 4°C. RNA precipitate formed a white gel-like pellet at the bottom of the tube. The supernatant was removed and the RNA pellet was mixed with 1ml 75% ethanol by vortexing. At this stage the RNA and ethanol mix was added 700µl at a time to a QIAamp spin column and this was centrifuged at 10,000 RPM for 15s at 4°C, discarding the flow through.

The QIAamp® RNA blood mini kit protocol was then joined at the stage of on column DNase digestion (6.3.4.a). The Extracted RNA was treated and stored as described.

6.3.5 Extraction of total RNA from peripheral blood mononuclear cells (PBMCs) using Polymorphprep™ and the RNeasy® mini kit

6.3.5.a   Extraction of peripheral blood mononuclear cells (PBMCs)

The extraction of PBMCs was commenced within 15 minutes of collecting venous blood in EDTA collection tubes.

Peripheral blood mononuclear cells (PBMCs) were selectively isolated from blood using a solution of sodium diatrizoate and dextran 500 (Polymorphprep™, Axis-Shield, Oslo, Norway, Catalogue number 1114683).

A 5ml volume of blood was carefully layered over 5mls Polymorphprep™ in a 15ml polypropylene tube (figure 6.1). Centrifugation was carried out at 2400 RPM for 45 minutes at 18°C. The top cell layer (PBMCs) was transferred to a fresh 15ml polypropylene tube placed on ice. PBMCs were washed with ice cold phosphate buffered saline (PBS, Invitrogen Corporation, California, USA), filling the tube. Centrifugation was performed at 1800 RPM for 5 minutes at 4°C and the supernatant was discarded. Cells were resuspended in PBS, the wash step was repeated and the supernatant was discarded once again. PBMC pellets were resuspended in 1ml of PBS and placed on ice.

Figure 6.1 Extraction of peripheral blood mononuclear cells (PBMCs). (A) 5mls of blood was carefully layered onto 5mls of polymorphprep. (B) After centrifugation. (C + D) The PBMC (top) cell layer and the polymorphonuclear (bottom) cell layer can be clearly seen.
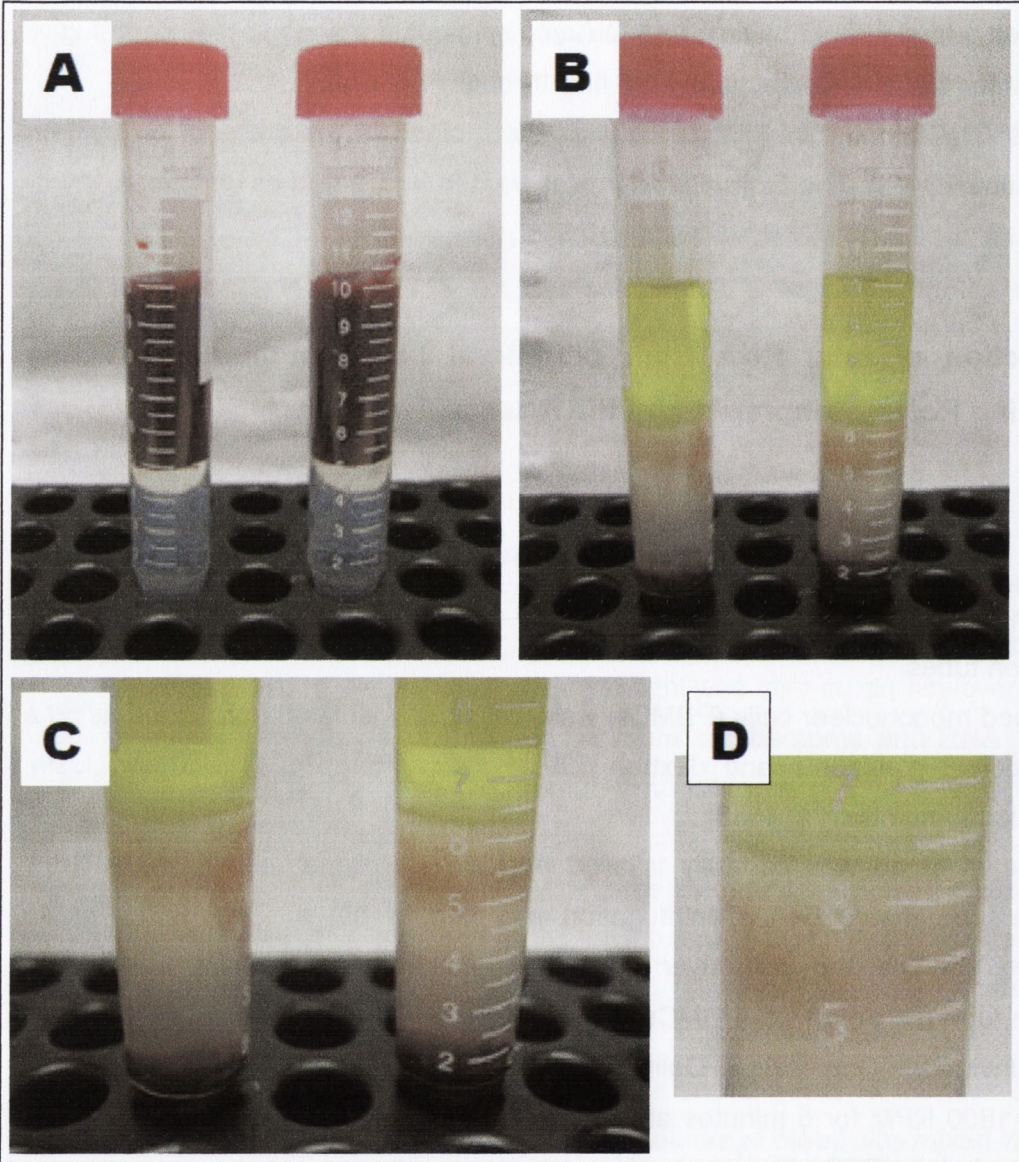
6.3.5.b Calculating the percentage viability and purity of the PBMC suspensions

Ethidium bromide / acridine orange solution was used to confirm the viability and purity of the PBMC suspensions. Ethidium bromide and acridine orange were both obtained from Sigma-Aldrich Ireland Ltd., Dublin, Ireland. A 10mg weight of each was added to 100mls PBS to make the solution.

A 10μl volume of each homogenous PBMC suspension was added to 20μl ethidium bromide / acridine orange solution. PBMCs in each sample were counted using fluorescent microscopy and a haemocytometer. PBMCs were distinguished by their morphology; PBMCs demonstrated single lobed nuclei (mononuclear) while polymorphonuclear leucocytes demonstrated multilobulated nuclei (figure 6.2). The percentage contamination of polymorphonuclear cells in the PBMC sample was calculated. The use of ethidium bromide / acridine orange solution resulted in live cells emitted green fluorescence, while dead cells emitted orange fluorescence. The percentage of live cells in the PBMC samples was calculated.

PBMC suspensions were divided into aliquots of 10×10^6 cells, in cryopreserve tubes. Centifugation was performed at 1800 RPM for 5 minutes at 4°C; the supernatant was then discarded. The first step of RNA extraction was commenced by adding 600μl buffer RLT with added β-Mercaptoethanol (RNeasy® mini kit component) to each cell pellet while stirring and mixing with the transfer pipette. Samples were then stored at -80°C.

Figure 6.2   The appearance of peripheral blood mononuclear cells (PBMCs) and polymorphonuclear cells when examined using fluorescent microscopy. (A) PBMCs demonstrated single lobed nuclei (mononuclear) while (B) polymorphonuclear cells demonstrated multilobulated nuclei (polymorphonuclear). The cells below are seen to emit green fluorescence and are viable.
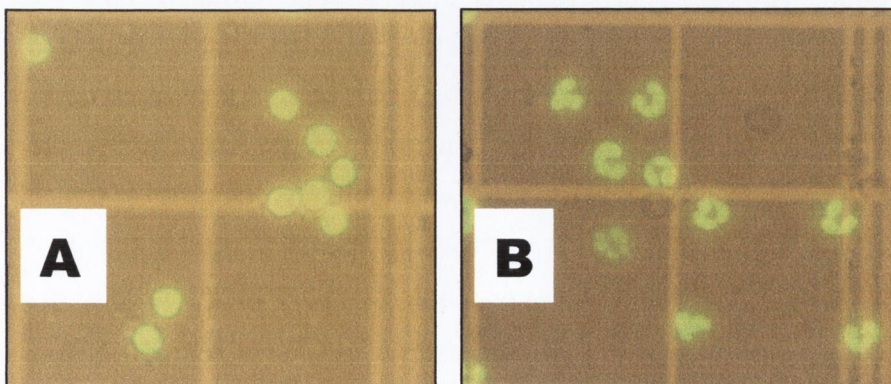
6.3.5.c   RNA Extraction from PBMC samples

RNA extraction was completed using the RNeasy® mini kit (Qiagen Ltd, West Sussex, United Kingdom, Catalogue number 74104). The following is a description of the protocol followed for samples; it is almost identical to that described for the QIAamp® RNA mini blood kit (section 3.6.4.a), except QIAamp spin columns are replaced by RNeasy mini columns. It is adapted from the manufacturers protocol (Qiagen, 2006b).

The PBMC samples with added buffer RLT were thawed on ice. Each sample was pipetted onto a QIAshredder homogenisation column and centrifugation was carried out at maximum speed (17,000 RPM) at 4°C for 3 minutes. This produced homogenised cell lysates which were collected in the 2ml collection tube of the QIAshredder column.

A 600µl volume (or volume equal to the lysate) of 70% ethanol was added to the cell lysates and mixed by pipetting. Up to 700µl of this mixture was added to an RNeasy mini column and this was centrifuged at 10,000 RPM for 15s at 4°C. The flow through into the collection tube was discarded. This step was repeated with the remaining cell lysate and ethanol mixture.

The optional on-column DNase digestion step was performed using RNase free DNase (Qiagen Ltd, Catalogue number 79254). Firstly, 350µl of wash buffer RW1 was pipetted onto the RNeasy mini column. Centrifugation was performed; 10,000 RPM for 15s at 4°C, discarding the flow through. The DNase working solution was formed by gently mixing 10µl DNase stock solution with 70µ buffer RDD. An 80µl volume of DNase working solution was pipetted directly onto the silica-gel membrane of the RNeasy mini column and placed at room temperature for 15 minutes. Finally, wash buffer RW1 was used to wash the RNeasy mini column as described before.

The RNeasy column was transferred to a new collection tube. A 500µl volume of buffer RPE (with added ethanol) was added to the column and left for 1 minute. Centrifugation was carried out at 10,000 RPM for 15s at 4°C, discarding the flow through. The column was washed for a second time with 500µl buffer RPE which was again left for 1 minute. Centrifugation at 10,000 RPM at 4°C was performed, this time for 2 minutes, again discarding the flow through. To ensure any residual ethanol was removed from the RNeasy mini column, this was then placed in a new collection tube and centrifugation was performed at 17,000 RPM at 4°C for one minute. This collection tube was then discarded.

To elute RNA from the RNeasy mini column, this was transferred to a 1.5ml cryopreserve collection tube. A 30µl volume of RNase-free water was pipetted onto the silica-gel membrane of the column and left for 3 minutes. The RNA was eluted by centrifugation at 10,000 RPM for 1 minute at 4°C. To obtain a higher concentration of RNA, this eluate was once again placed onto the membrane of the RNeasy mini column and left again for 3 minutes. Elution was then performed by centrifugation as before. The RNA samples were

immediately placed on ice, a 1µl aliquot was set aside to assess the quantity and purity of extracted RNA, and the RNA sample was stored at -80°C.

6.3.6 Assessing the concentration and purity of extracted RNA

The 1µl aliquot of RNA was used to assess the quantity and purity of extracted RNA using a NanoDrop® ND-1000 spectrometer (NanoDrop Technologies Inc., Delaware, USA). This provided the concentration of RNA in ng/µl (figure 6.3). It also provided ratios of the sample absorbance at 260nm and 280nm, the 260/280 ratio. A valve >1.9 indicated that the RNA sample was free from contaminants e.g. protein, and suitable to use for polymerase chain reaction (PCR).

Figure 6.3   Assessment of the concentration and purity of RNA samples using the NanoDrop spectrometer. This measures the concentration of RNA in ng/µl (181.2) and the 260/280 ratio of the sample (1.95).
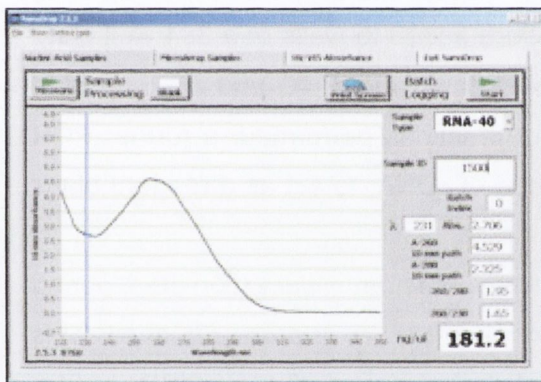
6.3.7 Generation of First-strand complimentary DNA (cDNA)

Complimentary DNA (cDNA) was made from extracted RNA using the SuperScript™ III Reverse Transcriptase kit (Invitrogen, catalogue number 18080-044). The volume of RNA samples containing 1µg of total RNA was calculated from the RNA concentrations obtained using the NanoDrop spectrometer. RNA samples were thawed on ice and 1µg of total RNA was added to 0.2ml tubes. The maximum volume of RNA which could be added to the tube was 11.67µl. A 0.33µl volume of 3µg/µl random primers (Invitrogen, catalogue number 48190-011) and 1µl of 10mM dNTP mix (Invitrogen, catalogue number 18427-013) were added. Nuclease free water was added to make the volume to 13µl. The 0.2ml tubes were then incubated at 65°C for 5 minutes using an ABI 2720 thermal cycler (Applied Biosystems, California, USA). A 4µl volume of 5X first-strand buffer, 1µl of 0.1M DTT, 1µl of 200U/µl SuperScript™ III Reverse Transcriptase (all kit components) and 1µl of 40U/µl RNaseOUT™ (Ribonuclease inhibitor, Invitrogen, catalogue number 10777-019)

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were added to the 0.2ml tubes and mixed by gentle pipetting. Samples were placed on the thermal cycler at 25°C for 5 minutes, 50°C for 60 minutes, 70°C for 15 minutes, and then held at 4°C until removal of samples. The cDNA generated was stored at -20°C until polymerase chain reaction (PCR) was performed.

6.3.8   Real time quantitative polymerase chain reaction (RQ PCR)

RQ PCR was performed using the Applied Biosystems 7900HT Fast Real-Time PCR System and TaqMan® gene expression assays. The manufacturer's guidelines were followed (Applied_Biosystems, 2007). The targets used were interleukin 10 (IL-10, Applied Biosystems, catalogue number Hs00174086_m1) and tumour necrosis factor alpha (TNF-α, Applied Biosystems, catalogue number Hs00174128_m1). Abelson murine leukemia viral oncogene homolog 1 (ABL, Applied Biosystems, catalogue number Hs00245443_m1) was used as the endogenous control.

After thawing the cDNA samples on ice, 10µl PCR reactions were performed: 1µl of cDNA, 0.5µl of pre-developed assay reagent (PDAR, i.e. IL-10, TNF-α or ABL), 3.5µl of nuclease free water and 5µl of TaqMan® universal PCR master mix, no AmpErase® UNG (Applied Biosystems, catalogue number 4324018) were added to wells of a 384 well plate (Applied Biosystems). Triplicate reactions were performed for IL-10 and TNF-α using duplicate ABL endogenous control. For each set of reactions performed, a further PCR reaction was performed alongside using water instead of cDNA, to act as a reagent blank / negative control.

The thermal cycler initially held samples at 95° for 10 minutes, an initialisation step, during which the cDNA is denatured, i.e. any secondary structures formed are removed. This was followed by 40 cycles of 95°C for 15 seconds followed by 1 minute at 60°C. At 95°, DNA is denatured, i.e. hydrogen bonds between complimentary DNA strands are disrupted to yield single strands of template DNA. Annealing and elongation then occur at 60°C, during which DNA polymerase attaches to DNA template strands and synthesises new DNA strands complementary to the template strands.

Real time quantification of DNA during each cycle was performed using Sequence Detection System (SDS) Software Version 2.1 (Applied Biosystems) (figure 6.4). For each of the 384 wells, the cycle where DNA amplification crossed the default threshold (C_T) was recorded.

Figure 6.4   Output of Sequence Detection System (SDS) Software showing the amplification plots for several PCR reactions. The red horizontal line on the graph represents the threshold amount of amplified target DNA. The C_T (threshold cycle) is the cycle number at which the amount of amplified target reaches the threshold; this is recorded by the software.
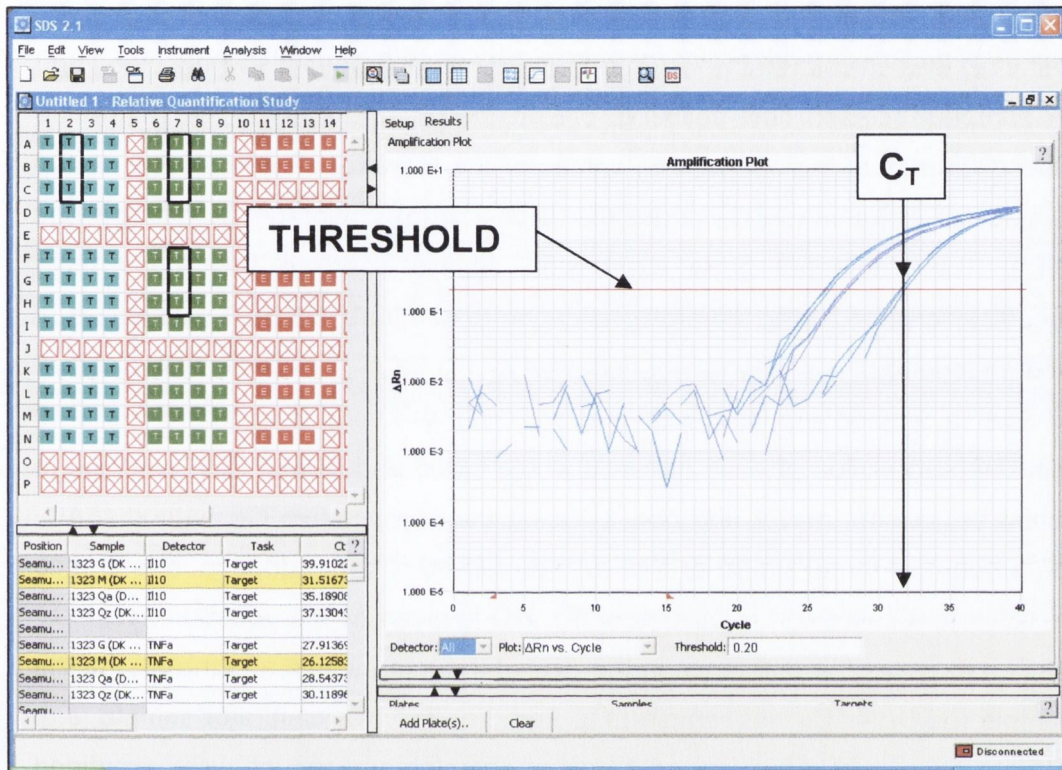
6.3.9 PCR data analysis

The raw data containing the C_T value for the PCR reactions was exported to Microsoft Office Excel 2003 software (Microsoft Corporation, Washington, USA). The average C_T value was calculated for the targets (IL-10 and TNF-α) and the endogenous control (ABL) for each sample using Excel software. A standard deviation of 0.5 was accepted when calculating the average C_T value; if the standard deviation was greater than 0.5, RQ PCR was repeated for the sample.

The ΔC_T (delta C_T) was calculated for IL-10 and TNF-α for each patient's whole blood and PBMC RNA samples at each time-point i.e. pre-op, pod 1 and pod 7. The ΔC_T value expresses the difference in the average C_T values of the target and endogenous control for each sample (ΔC_T = average C_T target (IL-10 or TNF-α) - average C_T endogenous control (ABL)). The TaqMan® gene expression assays used in this study have amplification efficiencies of 100%; therefore validation to compare the PCR efficiencies of the target and endogenous control was not necessary.

The comparative C_T method (Applied_Biosystems, 2007) was used to compare the IL-10 and TNF-α mRNA expression of each patient's whole blood and PBMC RNA samples at each time-point i.e. pre-op, pod 1 and pod 7. Using the comparative C_T method ($\Delta\Delta C_T$ method), the amount of target (IL-10 or TNF-α) normalised to an endogenous reference (ABL) and relative to a calibrator; relative quantity, RQ is given by the equation: RQ = $2^{-\Delta\Delta C_T}$. The $\Delta\Delta C_T$ expresses the difference in the ΔC_T of the test sample and the ΔC_T of the calibrator sample ($\Delta\Delta C_T$ = ΔC_T test sample (e.g. Pod 1 or 7) - ΔC_T calibrator sample (e.g. pre-op)). Differences in mRNA expression between samples and groups were examined by comparing the ΔC_T values and the relative quantity (RQ) of mRNA expression.

The Ratio of mRNA expression of pro-inflammatory TNF-α to anti-inflammatory IL-10 was also calculated using a comparative C_T method ($\Delta\Delta C_T$ method). The amount of TNF-α relative to IL-10 for each sample; relative quantity, RQ was given by the equation: RQ = $2^{-\Delta\Delta C_T}$. The $\Delta\Delta C_T$ value was the difference in the ΔC_T values of TNF-α and IL-10 for each sample ($\Delta\Delta C_T$ = ΔC_T TNF-α - ΔC_T IL-10).

6.3.10 Statistical Considerations

The primary endpoint of chapter 6 was to examine the overall cytokine immune response following oesophagectomy at an RNA level. Pre-op IL-10 and TNF-α mRNA expression levels in whole blood and PBMCs were compared with levels on pod 1 and pod 7. These were performed for the surgery and multimodal groups.

The second endpoint of chapter 6 was to compare the TNF-α and IL-10 mRNA cytokine responses following oesophagectomy in patients treated with surgery alone or a multimodal treatment regimen. Whole blood and PBMC, IL-10 and TNF-α mRNA expression levels were compared between groups, overall using an area under the curve

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analysis (AUC) and at pre-op, pod 1 and pod 7 time points. The ratio of mRNA expression of pro-inflammatory TNF-α to anti-inflammatory IL-10 was also compared between groups at these time points.

Statistical analysis was performed using SPSS version 16.0 (Statistical Package for the Social Sciences, SPSS inc., Illinois, USA). Parametric analysis was performed. Cytokine data between days for each group was compared using the paired samples T-test. Cytokine data between the surgery and multimodal groups was compared using the independent samples T-test. Categorical data between groups was compared using the Pearson Chi-square test or Fisher's exact test. A p value < 0.05 was considered statistically significant and all tests performed were two-tailed tests.

The number of patients or replication (r) required in each treatment group was estimated using the formula: $r = 16 (CV / d)^2$. CV, the coefficient of variation was taken as 20%, usual for biological experiments. A type I error of 5% and a type II error of 20% were assumed. Changes in mRNA expression levels were expected to be greater than changes in serum cytokine levels. Therefore in the PCR study, a 30% difference (d) in mRNA expression levels was considered important not to miss between the groups (compared to a 25% difference in chapter 5). The minimum number of patients needed in each group for the PCR study was therefore 8.

6.4 RESULTS

6.4.1 Patients recruited

Eighteen of the 31 patients recruited in chapter 5 were studied in chapter 6, of which 8 (44%) received surgery alone (surgery group) and 10 (56%) received a multimodal treatment protocol (multimodal group). The serum cytokine study (chapter 5) commenced before the PCR cytokine study (chapter 6). After commencement of this PCR study, all patients were enrolled into both serum and PCR studies. All 18 patients had blood collected preoperatively (pre-op) and on days 1 and 7 postoperatively (pod 1 and pod 7). Four of the 10 multimodal patients had blood collected before commencing preoperative chemoradiotherapy (pre-CRT). Patients had frequently commenced chemoradiotherapy when recruited to take part in this study.

The patient characteristics, operative parameters, pathological parameters and postoperative course including the occurrence of complications and hospital stay are shown for all patients in each group (table 6.1). The postoperative complications which occurred are presented in further detail for all patients (table 6.2).

Table 6.1   Patient   characteristics,   operative   parameters,   pathological parameters and postoperative course of patients in both surgery and multimodal treatment groups

Group	Patient	Age (years)	Gender	Smoker	BMI (kg/m²)	ASA grade	T stage	N stage	Stage	Morphology	TRG	Operation	Blood loss (mls)	Transfusion (units)	Op time (hrs)	Complication	Death	HDU / ICU (days)	Hosp stay (days)
S	S1	78	M	Never	26.8	2	pT3	pN0	2a	Adeno		2 stage	2775	0	5	Yes	yes	34	34
S	S2	58	M	Current	23.5	2	pT0	pN0	0	HGD		2 stage	1400	0	7.5			4	24
S	S3	67	M	Ex	26.3	2	pT3	pN1	3	Adeno		2 stage	3000	0	4.5	Yes		2	29
S	S4	63	M	Current	17.4	2	pT3	pN1	3	Adeno		2 stage	2000	4	7	Yes		11	34
S	S5	62	M	Never	26.0	2	pT3	pN1	3	Adeno		2 stage	500	0	4			3	16
S	S6	68	F	Never	18.4	2	pT1	pN0	1	Squamous		3 stage	450	0	5.25	Yes		8	30
S	S7	71	M	Never	21.7	2	pT3	pN1	3	Adeno		2 stage	1700	0	5.5	Yes		1	48
S	S8	60	M	Never	30.7	2	pTis	pN0	0	Adeno		2 stage	355	0	6	Yes		4	23
M	M1	58	M	Ex	40.5	1	pT2	pN1	2b	Adeno	3	2 stage	2500	0	5.5	Yes		5	22
M	M2	63	M	Never	26.2	2	pT3	pN1	3	Adeno	3	2 stage	1040	0	4.5	Yes		3	27
M	M3	72	F	Never	29.1	3	pT3	pN0	2a	Adeno	3	3 stage	390	0	4	Yes		3	21
M	M4	72	M	Never	29.4	1	pT3	pN1	3	Adeno	3	2 stage	600	2	7			3	15
M	M5	57	M	Never	26.5	2	pT4	pN1	3	Squamous	4	3 stage	800	0	5			3	16
M	M6	65	M	Ex	27.1	2	pTx	pN0	2a	Adeno	1	3 stage	2200	1	6	Yes	yes	15	17
M	M7	37	M	Never	26.9	2	pT0	pN1	2b	Adeno	2	2 stage	1500	0	4			4	13
M	M8	62	M	Ex	33.3	1	pT2	pN1	2b	Adeno	3	2 stage	500	0	5			2	19
M	M9	58	F	Ex	26.8	1	pT0	pN0	0	Squamous	1	3 stage	200	2	4	Yes		4	19
M	M10	54	M	Ex	23.4	1	pT3	pN1	3	Adeno	3	2 stage	1285	0	5.5			3	20

S = Surgery, M = Multimodal, BMI = Body Mass Index, ASA = American Society of Anesthesiologists, HGD = High Grade Dysplasia TRG = Tumour Regression Grade, Op = Operation, HDU = High Dependency Unit, ICU = Intensive Care Unit, Hosp = Hospital

Table 6.2 Detailed description of the postoperative complications which occurred in both surgery and multimodal treatment groups

Group	Patient	Complication	Mortality (days post op)
S	S1	Pleural effusion, Pneumothorax, Myocardial infarction, Heart failure, Renal failure, Prolonged intubation	34 days
S	S2		
S	S3		
S	S4	Pleural effusion, Pneumothorax, Chylothorax	
S	S5		
S	S6	Pleural effusion	
S	S7	Pleural effusion, Anastomotic Leak (radiographic), Hydropneumothorax	
S	S8	Atelactasis, Atrial fibrillation	
M	M1	Atelactasis	
M	M2	Deep venous thrombosis	
M	M3	Central line infection	
M	M4		
M	M5		
M	M6	Pneumonia, Prolonged intubation, Renal failure, Central line Infection	15 days
M	M7		
M	M8		
M	M9	Pleural effusion	
M	M10		

S = Surgery, M = Multimodal

6.4.2 Comparability of the surgery and multimodal groups

The 8 surgery and 10 multimodal patients were compared in terms of characteristics, operative parameters, tumour pathology and complication rates (table 6.3).

There was a statistically significant increased body mass index (BMI) in the multimodal group compared to the surgery group (mean BMI: surgery group 24kg/m^2, multimodal group 29kg/m^2, p = 0.037). There were no other differences found between groups when the age, number of female patients, smoking history (current or ex smokers) or ASA grade were examined. All tumours in the patients studied in chapter 6 were located in the lower oesophagus or at the oesophago-gastric junction (OGJ). Not surprising therefore, only 3 patients (1 patient in the surgery group and 2 patients in the multimodal group) had a squamous cell carcinoma on histological examination, the rest being adenocarcinoma. In the surgery group one patient had Barrett's oesophagus with high grade dysplasia. All patients were treated with curative intent and had a laparotomy and thoracotomy performed during surgical resection; a 2 stage oesophagectomy. One patient in the surgery group and 4 patients in the multimodal group had a 3 stage oesophagectomy (an additional neck incision) (p = 0.314). The operating time and blood loss were comparable in each group. No significant differences were found on pathological examination with respect to tumour stage, or the presence of lymph node metastasis.

The rate of postoperative complications was similar in both groups of patients when examined overall and when examined with the exclusion of an isolated pleural effusion. There were no significant differences when complications were analysed in terms of respiratory complications and non-respiratory complications. The length of stay in the high dependency unit or intensive care unit was not found to be different between groups. However, the postoperative length of stay in hospital was significantly greater in the surgery group than the multimodal group (mean 30 days in the surgery group versus 19 days in the multimodal group, p = 0.005).

One patient in each group died in hospital after surgery. In the surgery group this death occurred at 34 days after surgery, complicated by a pleural effusion, pneumothorax, myocardial infarction, heart failure, renal failure and prolonged intubation. In the Multimodal group a death occurred 15 days post-op following pneumonia, a central line Infection, renal failure and prolonged intubation.

Table 6.3   Comparison of the surgery and multimodal groups in terms of patient characteristics, operative parameters, tumour pathology and complication rates

	Surgery (n = 8)	Multimodal (n = 10)	p
<u>Patient and operative parameters</u>			
Mean age (yrs) (Std. Error Mean)	66 (2)	60 (3)	0.160
Gender			
Male	7	8	1.000
Female	1	2	
Smoker			
Current or ex smoker	3	5	1.000
No	5	5	
Mean BMI (kg/m2) (Std. Error Mean)	24 (2)	29 (2)	0.037
ASA grade			
I - II	8	9	0.358
III - IV	0	1	
Mean operation time (hrs) (Std. Error Mean)	5.7 (0.6)	4.9 (0.4)	0.257
Mean blood loss (mls) (Std. Error Mean)	1497 (424)	1108 (273)	0.435
Mean blood transfusion (units) (Std. Error Mean)	0.5 (0.5)	0.5 (0.5)	1.000
Operation type			
2 stage	7	6	0.314
3 stage	1	4	
Mean HDU / ICU stay (days) (Std. Error Mean)	8 (4)	5 (1)	0.306
Mean post-op hospital stay (days) (Std. Error Mean)	30 (3)	19 (1)	0.005
<u>Pathological analysis</u>			
Tumour histology			
Squamous cell	1	2	1.000
Adeno	5	8	
HGD / Intramucosal	2	0	
Pathologic tumour (T) stage			
T0 - T2	3	5	0.664
T3 - T4	5	5	
Pathologic nodal (N) stage			
N0	4	3	0.630
N1	4	7	
Tumour Regression Grade (TRG) (Multimodal group)			
TRG 1- 2		3	
TRG 3		6	
TRG 4- 5		1	
<u>Complications</u>			
No Complication	3	5	0.664
Complication	5	5	
Complication excluding isolated pleural effusion	4	4	1.000
Respiratory complication	5	3	0.342
Non respiratory complication	3	3	1.000
Mortality	1	1	1.000

BMI = Body Mass Index, ASA = American Society of Anesthesiologists
HDU = High Dependency Unit, ICU = Intensive Care Unit, HGD = High Grade Dysplasia

6.4.4 Serum results for patients studied in PCR study

The Serum cytokine and growth factor results obtained in the serum study (chapter 5) are shown for the 8 surgery and 10 multimodal patients also studied in the PCR study (chapter 6) (table 6.4). The mean (and standard error of mean in parenthesis) serum levels expressed in pg/ml, pre-op and on pod 1 and pod 7 are shown for both study groups. Pre-CRT levels are also shown for 4 of the 10 multimodal patients (4 multimodal patients had pre-CRT serum collected). Significant differences in cytokine levels on pod 1 and pod 7 compared to pre-op levels are indicated.

When the perioperative serum cytokine and growth factor levels were analysed for the 8 surgery and 10 multimodal patients studied in the PCR study, the results were similar to the results obtained for all patients studied in the serum study.

There were significant rises in serum IL-6, IL-8, IL-10 and MCP1 levels on pod 1 (for IL-10 this was significant in the surgery group but not in the multimodal group). There was a rise in serum VEGF levels by pod 7 in the surgery group; in the multimodal group this did not reach statistical significance.

For the other 7 cytokines measured (IL-2, IL-4, IFN-γ, TNF-α, IL-1α, IL-1β and EGF), there was no significant change in the postoperative levels in either group.

No significant differences were found between the surgery and multimodal groups, overall using an area under the curve (AUC) analysis, or at any individual time point, for all 12 cytokines and growth factors analysed.

To summarise, the serum cytokine results for the 18 patients studied in the PCR study (chapter 6) were similar to the results obtained for all 31 patients in the serum study (chapter 5). The 18 patients studied in the PCR study are therefore representative of the cohort of 31 patients studied in the serum study.

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Table 6.4 Mean and (standard error of mean) serum cytokine and growth factor levels pre-op, on pod 1, pod 7, and pre-CRT for the 8 surgery and 10 multimodal patients studied in chapter 6. Significant differences in cytokine levels on pod 1, pod 7 and pre-CRT compared to pre-op levels are indicated.

Surgery Group

Cytokine	Unit	pre-op (n = 8)		pod 1 (n = 8)		pod 7 (n = 8)	
IL-2	pg/ml	30	(24)	19	(10)	40	(29)
IL-4	pg/ml	1	(1)	3	(2)	2	(1)
IL-6	pg/ml	32	(20)	302 ‡	(24)	112	(48)
IL-8	pg/ml	18	(4)	50 ‡	(8)	29	(5)
IL-10	pg/ml	1	(1)	3 †	(1)	1	(0)
VEGF	pg/ml	151	(43)	146	(27)	410 †	(123)
IFN-γ	pg/ml	2	(1)	1	(1)	2	(1)
TNF-α	pg/ml	6	(2)	9	(3)	7	(2)
IL-1α	pg/ml	2	(1)	2	(1)	3	(1)
IL-1β	pg/ml	0	(0)	1	(0)	1	(0)
MCP1	pg/ml	386	(44)	688 †	(123)	593 †	(101)
EGF	pg/ml	117	(25)	97	(18)	82	(20)

† p < 0.05 versus pre-op, ‡ p < 0.01 versus pre-op
No differences between Surgery and Multimodal groups

Multimodal Group

Cytokine	Unit	pre-CRT (n = 4)		pre-op (n = 10)		pod 1 (n = 10)		pod 7 (n = 10)	
IL-2	pg/ml	11	(6)	11	(5)	10	(5)	10	(4)
IL-4	pg/ml	3	(1)	1	(1)	3	(1)	2	(1)
IL-6	pg/ml	12	(2)	23	(11)	283 ‡	(27)	38 ‡	(8)
IL-8	pg/ml	29	(8)	14	(4)	52 †	(13)	34 †	(8)
IL-10	pg/ml	0	(0)	0	(0)	1	(0)	0	(0)
VEGF	pg/ml	109	(10)	122	(25)	118	(24)	267	(92)
IFN-γ	pg/ml	7	(3)	8	(6)	1	(1)	3	(1)
TNF-α	pg/ml	7	(1)	7	(1)	6	(1)	8	(1)
IL-1α	pg/ml	5	(3)	2	(1)	4	(2)	4	(2)
IL-1β	pg/ml	2	(1)	1	(0)	1	(0)	1	(0)
MCP1	pg/ml	470	(57)	472	(43)	648 †	(72)	616 †	(78)
EGF	pg/ml	93	(29)	145	(28)	108	(23)	105	(26)

† p < 0.05 versus pre-op, ‡ p < 0.01 versus pre-op
No differences between Surgery and Multimodal groups

6.4.5 Quality control; the cell viability, RNA concentration and RNA purity for extracted RNA.

The mean viability of isolated peripheral blood mononuclear cells (PBMCs) using Polymorphprep™ was 98% (standard error of mean 0.55%); the mean purity was also 98% (standard error of mean 0.39%).

The mean (and standard error of mean in parenthesis) measures of the quantity and quality control of the RNA extracted from whole blood and PBMCs at each time point are shown (table 6.5). For cDNA synthesis, we aimed to use 1µg of total RNA. For RNA extracted from whole blood, the mean amount of RNA used for cDNA synthesis (for all Surgery and Multimodal patients) was 0.9µg pre-CRT, 0.8µg pre-op and 1µg at other time-points. For RNA extracted from PBMCs, the mean amount of RNA used for cDNA synthesis was 1µg pre-CRT, 0.7µg pre-op and 0.9µg at other time-points. The starting amount of cDNA is not crucial when performing RQ PCR because target gene expression is normalised to an endogenous control rather than the amount of cDNA used. The mean 260/280 ratio of extracted RNA was consistently greater than 1.9 (mean 2.1 for samples at all time-points), confirming its purity and suitability for use in PCR.

PCR reactions using water instead of cDNA were performed alongside each set of targets or controls for each sample, to serve as a reagent blank / negative control.

Table 6.5 Quality control: Mean and (standard error of mean) measures of the quantity (amount RNA used and RNA concentration) and quality (260/280 ratio) of RNA extracted. The values are shown for each extraction method at each time point.

Surgery Group

RNA source	Extraction	Varible	Unit	pre-op	pod 1	pod 7
Whole Blood	QIAamp® (N = 7)	Amount RNA	(µg)	0.8 (0.1)	1.0 (0.0)	1.0 (0.0)
		Conc. RNA	(ng/µl)	74 (12)	222 (43)	333 (160)
		260/280 ratio		2.2 (0.1)	2.1 (0.0)	2.1 (0.0)
PBMC	Polymorphprep™ & RNeasy® mini (N = 8)	Amount RNA	(µg)	0.8 (0.1)	0.9 (0.1)	0.0 (0.2)
		Conc. RNA	(ng/µl)	169 (36)	218 (40)	428 (22*)
		260/280 ratio		2.2 (0.1)	2.1 (0.0)	2.1 (0.1)
Blood	QIAzol® (N = 1)	Amount RNA	(µg)	0.5	1.0	0.8
		Conc. RNA	(ng/µl)	45	104	68
		260/280 ratio		2.1	2.1	2.1

Multimodal Group

RNA source	Extraction	Varible	Unit	pre-CRT	pre-op	pod 1	pod 7
Whole Blood	QIAamp® (N = 9, pre-CRT = 4)	Amount RNA	(µg)	0.9 (0.1)	0.8 (0.1)	1.0 (0.0)	1.0 (0.0)
		Conc. RNA	(ng/µl)	113 (32)	89 (25)	152 (16)	128 (16)
		260/280 ratio		2.1 (0.0)	2.1 (0.0)	2.1 (0.0)	2.1 (0.0)
PBMC	Polymorphprep™ & RNeasy® mini (N = 10, pre-CRT = 4)	Amount RNA	(µg)	1.0 (0.0)	0.6 (0.1)	0.8 (0.1)	0.9 (0.0)
		Conc. RNA	(ng/µl)	163 (42)	79 (22)	156 (45)	208 (43)
		260/280 ratio		2.1 (0.0)	2.1 (0.0)	2.1 (0.0)	2.1 (0.0)
Blood	QIAzol® (N = 1, pre-CRT = 0)	Amount RNA	(µg)		0.4	1.0	1.0
		Conc. RNA	(ng/µl)		35	108	184
		260/280 ratio			2.1	2.1	2.1

6.4.6 Perioperative changes in whole blood and PBMC, IL-10 and TNF-α mRNA expression in the surgery and multimodal groups

Perioperative IL-10 and TNF-α mRNA expression levels in whole blood and PBMCs were analysed for the surgery and multimodal groups by comparing the ΔC_T and RQ values on pod 1, pod 7 and pre-CRT (multimodal group) with the pre-op value. The mean (standard error of mean) ΔC_T values of IL-10 and TNF-α for whole blood and PBMCs, at each time point are shown (table 6.6). The mean (standard error of mean) RQ values of IL-10 and TNF-α for whole blood and PBMCs, at each time point are also shown (table 6.7). Figure 6.5 provides a graphic illustration of the perioperative RQ values of IL-10 and TNF-α. Significant differences in ΔC_T or RQ values on pod 1, pod 7 and pre-CRT compared to pre-op are indicated for each group.

Whole blood IL-10 mRNA expression levels increased significantly on the first postoperative day (pod 1) compared to pre-op levels in surgery and multimodal patients. The mean relative quantity (RQ) of mRNA expression on day 1 in the surgery group was 33, p = 0.017 and in the multimodal group was 38, p = 0.015. The RQ values are expressed relative to the pre-op expression which therefore has a relative quantity (RQ) of 1.

Postoperative changes in PBMC IL-10 mRNA expression were similar; significant increases were found on pod 1 compared to pre-op in both groups (mean RQ on pod 1: surgery group 29, p = 0.004 and multimodal group 19, p = 0.006). Once again the pre-op RQ value was 1. Expression levels remained significantly elevated in the surgery group on pod 7 (mean RQ: surgery group 3.9, p = 0.015).

Whole blood TNF-α mRNA expression levels were not found to be different on pod 1 or pod 7 compared to baseline pre-op levels in either the surgery or multimodal study groups.

PBMC TNF-α mRNA expression levels increased in the surgery group only to a mean RQ of 1.9 on pod 1 compared to a mean pre-op RQ of 1, p = 0.036.

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Table 6.6   Mean and (standard error of mean) whole blood and PBMC IL-10, TNF-α and ratio of TNF-α: IL-10 mRNA expression for the surgery and multimodal groups, pre-op, on pod 1, pod 7 and pre-CRT. Delta C_T (ΔC_T) values of targets, IL-10 and TNF-α normalised to the endogenous control, ABL are shown. The ΔC_T of TNF-α - ΔC_T of IL-10 was used to express the ratio of TNF-α: IL-10 mRNA expression. Differences in mRNA expression levels on pod 1, pod 7 and pre-CRT compared to pre-op levels are indicated.

Surgery Group

Cytokine	Source of RNA	Unit	pre-op (N = 8)		pod 1 (N = 8)		pod 7 (N = 8)	
IL-10	Whole Blood	ΔC_T	2.5	(0.3)	-2.0 ‡	(0.6)	1.1 †	(0.6)
	PBMC	ΔC_T	3.1	(0.3)	-1.5 ‡	(0.4)	1.4 ‡	(0.4)
TNF-α	Whole Blood	ΔC_T	-3.0 #	(0.2)	-4.0 †	(0.5)	-3.1	(0.3)
	PBMC	ΔC_T	-2.0 ##	(0.2)	-2.8 †	(0.4)	-1.2 #	(0.3)
TNF-α: IL10	Whole Blood	ΔC_TTNF-α - ΔC_T IL-10	-5.5	(0.4)	-1.9 ‡	(0.4)	-4.1 †	(0.5)
	PBMC	ΔC_TTNF-α - ΔC_T IL-10	-5.1	(0.3)	-1.3 ‡	(0.3)	-2.6 ‡	(0.3)

† p < 0.05 versus pre-op. ‡ p < 0.01 versus pre-op
# p < 0.05 Surgery Vs Multimodal ## p < 0.01 Surgery Vs Multimodal
No differences between Surgery and Multimodal groups (AUC Analysis)

Multimodal Group

Cytokine	Source of RNA	Unit	pre-op (N = 10)		pod 1 (N = 10)		pod 7 (N = 10)		pre-CRT (N = 4)	
IL-10	Whole Blood	ΔC_T	2.0	(0.3)	-2.5 ‡	(0.4)	0.7	(0.5)	2.3	(0.7)
	PBMC	ΔC_T	2.0	(0.5)	-1.7 †	(0.5)	0.3	(0.8)	2.4	(0.7)
TNF-α	Whole Blood	ΔC_T	-4.0 #	(0.3)	-4.5	(0.2)	-3.9	(0.3)	-3.0	(0.3)
	PBMC	ΔC_T	-3.1 ##	(0.3)	-3.3	(0.3)	-2.9 #	(0.5)	-2.3 ‡	(0.3)
TNF-α: IL10	Whole Blood	ΔC_TTNF-α - ΔC_T IL-10	-6.0	(0.4)	-2.0 †	(0.3)	-4.6 †	(0.4)	-5.3	(0.5)
	PBMC	ΔC_TTNF-α - ΔC_T IL-10	-5.1	(0.4)	-1.6 †	(0.4)	-3.2 ‡	(0.4)	-4.7	(0.9)

† p < 0.05 versus pre-op. ‡ p < 0.01 versus pre-op
# p < 0.05 Surgery Vs Multimodal ## p < 0.01 Surgery Vs Multimodal
No differences between Surgery and Multimodal groups (AUC Analysis)

ΔC_T = average C_T target (IL-10 or TNFA) - average C_T endogenous control (ABL). C_T = cycle where DNA amplification crosses treshold amount
AUC analysis = area under the curve analysis

Table 6.7   Mean and (standard error of mean) relative quantity (RQ) of whole blood and PBMC, IL-10, TNF-α and ratio of TNF-α: IL-10 mRNA expression for the surgery and multimodal groups, pre-op, on pod 1, pod 7 and pre-CRT. For IL-10 and TNF-α, the expression on pod 1, pod 7 and pre-CRT is expressed as a relative quantity (RQ) of the pre-op level (pre-op level is 1). For TNF-α: IL-10 expression, TNF-α is expressed as a relative quantity (RQ) of IL-10 expression. Significant differences in expression levels on pod 1, pod 7 and pre-CRT compared to pre-op levels are indicated.

Surgery Group

Cytokine	Source of RNA	Unit	pre-op (N = 8)	pod 1 (N = 8)	pod 7 (N = 8)
IL-10	Whole Blood	RQ	1 (0)	33 † (10.2)	3.8 (1.2)
	PBMC	RQ	1 (0)	29 ‡ (6.6)	3.9 † (0.9)
TNF-α	Whole Blood	RQ	1 (0)	2.6 (0.9)	1.3 (0.3)
	PBMC	RQ	1 (0)	1.9 † (0.4)	0.7 (0.2)
TNF-α: IL-10	Whole Blood	RQ	55 (14)	4.7 ‡ (1.1)	25 (8.6)
	PBMC	RQ	39 (8)	2.7 ‡ (0.5)	7.0 ‡ (1.3)

† $p < 0.05$ versus pre-op   ‡ $p < 0.01$ versus pre-op

Multimodal Group

Cytokine	Source of RNA	Unit	pre-op (N = 10)	pod 1 (N = 10)	pod 7 (N = 10)	pre-CRT (N = 4)
IL-10	Whole Blood	RQ	1 (0)	38 † (12.5)	10.1 (7.5)	0.8 (0.2)
	PBMC	RQ	1 (0)	19 ‡ (5.0)	14.0 (8.0)	1.3 (0.5)
TNF-α	Whole Blood	RQ	1 (0)	1.6 (0.3)	1.1 (0.2)	0.6 (0.2)
	PBMC	RQ	1 (0)	1.2 (0.2)	1.3 (0.4)	0.7 ‡ (0.0)
TNF-α: IL-10	Whole Blood	RQ	86 (21)	4.9 † (1.0)	31 † (8.2)	48 (21)
	PBMC	RQ	58 (27)	4.4 (1.7)	13 (3.5)	39 (17.0)

† $p < 0.05$ versus pre-op   ‡ $p < 0.01$ versus pre-op

RQ (for IL-10 and TNF-α) = Relative Quantity of mRNA expression, relative to pre-op level
RQ (for TNF-α: IL-10) = Relative Quantity of TNFA mRNA expression, relative to IL-10 mRNA expression

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Figure 6.5 (Following three pages)

Perioperative relative quantity (RQ) of whole blood and PBMC IL-10, TNF-α and TNF-α: IL-10 mRNA expression for the surgery group (closed boxes and continuous error bars) and multimodal group (open boxes and interrupted error bars). The graphs display the mean mRNA expression at pre-CRT, pre-op, pod 1 and pod 7 time-points, error bars show the standard error of the mean. The relative quantity (RQ) of IL-10 and TNF-α mRNA expression is relative to pre-op level. The relative quantity (RQ) of TNF-α: IL-10 mRNA expression is relative to the IL-10 level. There were no significant overall differences (p-values shown) between the surgery and multimodal groups using an area under the curve (AUC) analysis.

Figure 6.5 (Continued)

Whole blood IL-10 mRNA Expression
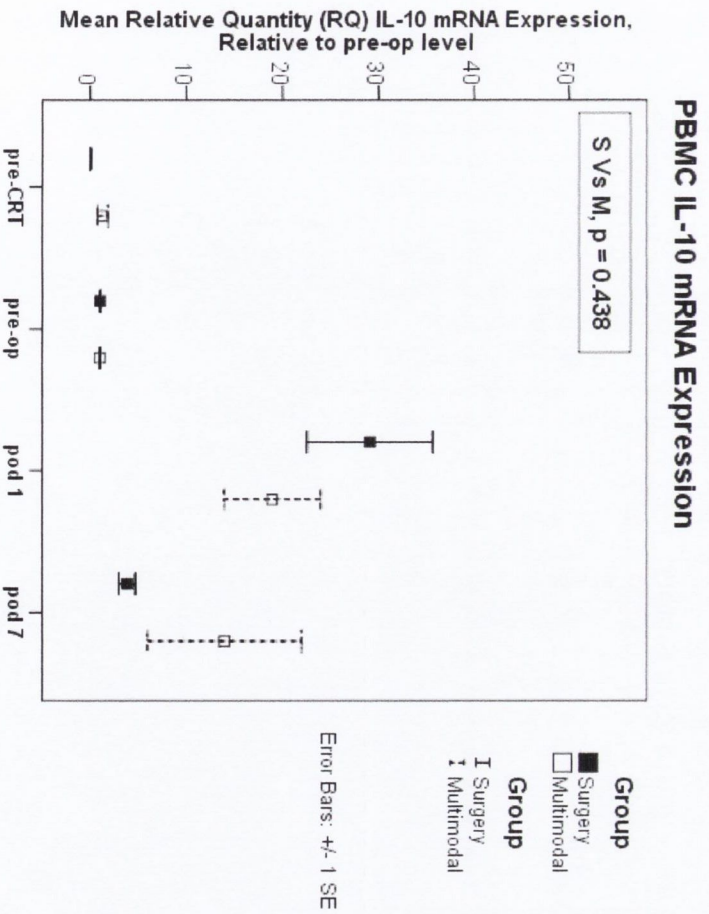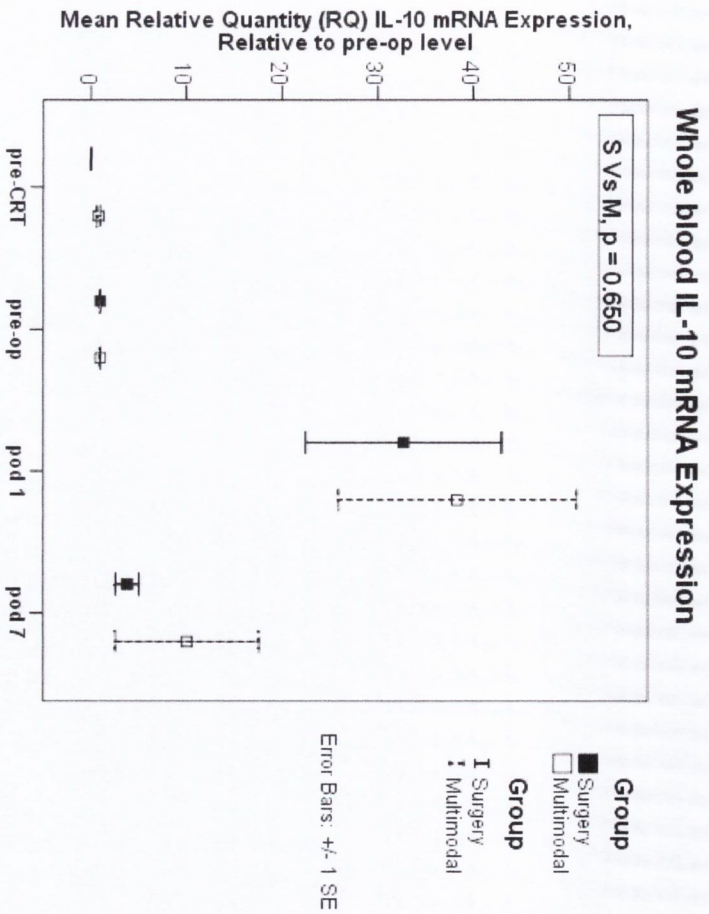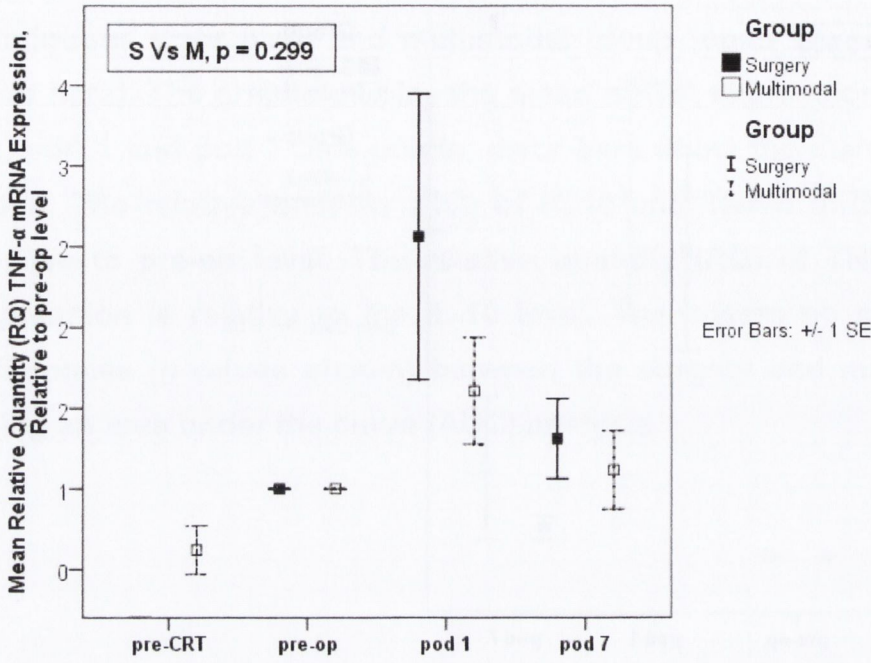
PBMC IL-10 mRNA Expression

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Figure 6.5 (Continued)

Whole blood TNF-α mRNA Expression

PBMC TNF-α mRNA Expression

Figure 6.5 (Continued)

Whole blood TNF-α: IL-10 mRNA Expression

PBMC TNF-α: IL-10 mRNA Expression
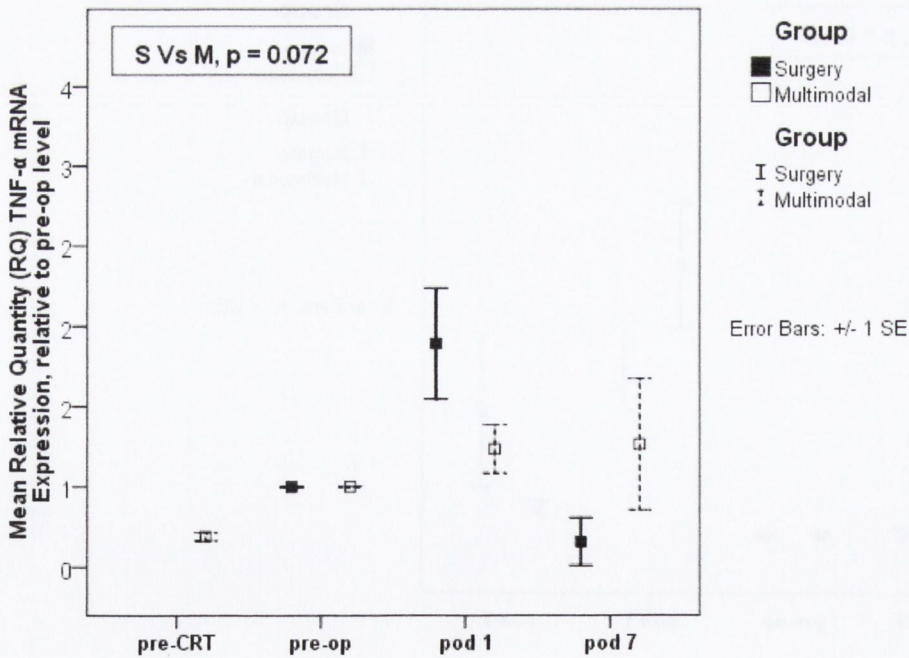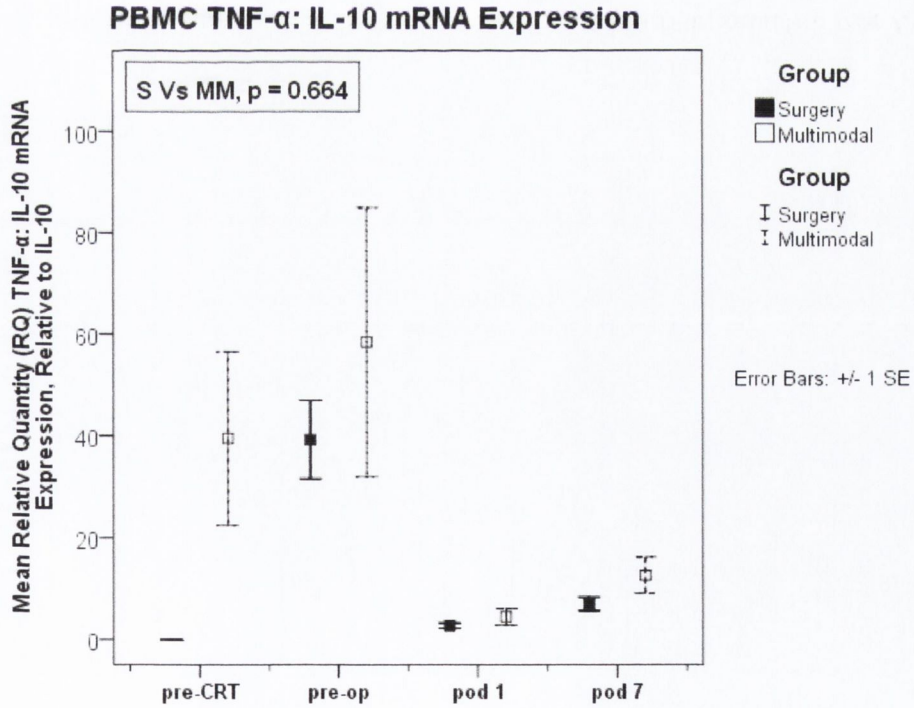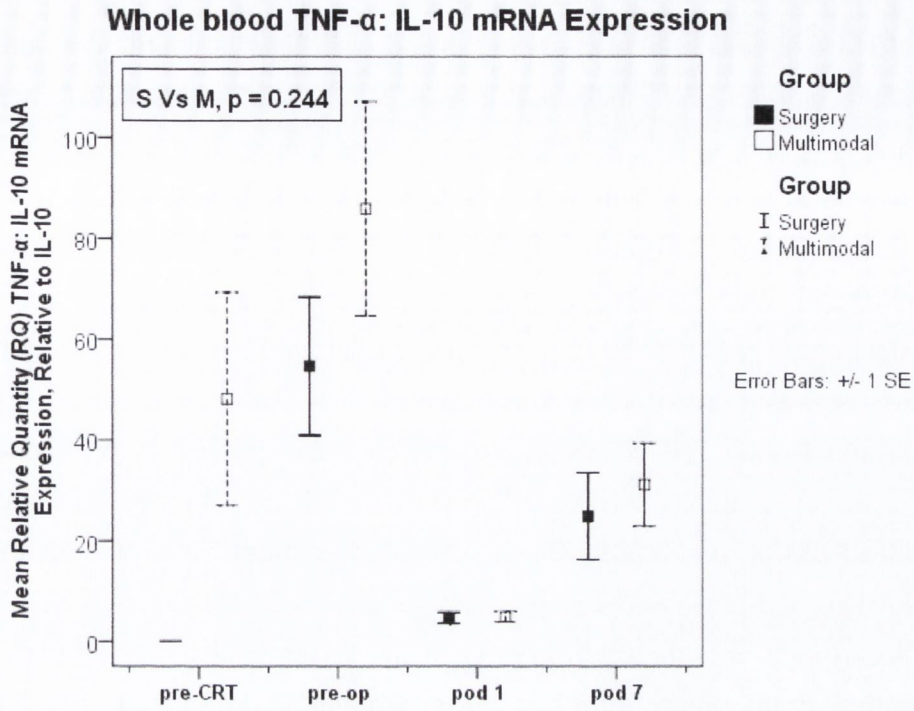
6.4.7 Comparison of whole blood and PBMC, IL-10 and TNF-α mRNA expression following oesophagectomy in surgery and multimodal patients

IL-10 and TNF-α mRNA expression levels in whole blood and PBMCs, were compared between patients in the surgery and multimodal groups overall and at individual time-points (tables 6.6 and 6.7, and figure 6.5). Mean ΔC_T values were used to calculate a mean relative quantity (RQ) of whole blood and PBMC, IL-10, TNF-α and TNF-α: IL-10 mRNA expression in the multimodal group, relative to the level in the surgery group at each time-point (table 6.8).

No differences in whole blood or PBMC IL-10 expression were found between the surgery and multimodal groups at pre-op, pod1 or pod7 time-points.

Pre-op whole blood and PBMC TNF-α mRNA expression were greater in multimodal patients compared to surgery patients, corresponding to mean RQs of 2.1 for whole blood, $p = 0.012$ and 2.2 for PBMCs, $p = 0.009$. These compared to mean RQs of 1 for the surgery group.

PBMC TNF-α mRNA expression was again greater in the multimodal group on pod 7 compared to the surgery group, mean RQ 3.2 in the multimodal group Vs mean RQ 1 in the surgery group, $p = 0.030$.

However, comparing overall IL-10, TNF-α and TNF-α: IL-10 mRNA expression using an area under the curve (AUC) analysis, there were no significant overall differences between the surgery and multimodal groups (Figure 6.5, overall p values are shown).

Table 6.8 Mean relative quantity (RQ) of whole blood and mononuclear cell (PBMC), IL-10, TNF-α and TNF-α: IL-10 mRNA expression, pre-op and on pod 1 and pod 7, relative to the level in the surgery group. The mean mRNA expression in the multimodal group is expressed as a relative quantity (RQ) of the level in the surgery group (level in surgery group is 1). The mean RQ values are calculated from the mean ΔCT values. Significant differences in the ΔCT levels between the surgery and multimodal groups are indicated.

Cytokine	Source of RNA	Unit	Pre-op Surgery (N = 8)	Pre-op Multimodal (N = 10)	Pod 1 Surgery (N = 8)	Pod 1 Multimodal (N = 10)	Pod 7 Surgery (N = 8)	Pod 7 Multimodal (N = 10)
IL-10	Whole Blood	Mean RQ (rel. surgery)	1	1.4	1	1.4	1	1.3
	PBMC	Mean RQ (rel. surgery)	1	2.1	1	1.1	1	2.1
TNF-α	Whole Blood	Mean RQ (rel. surgery)	1	2.1 #	1	1.5	1	1.8
	PBMC	Mean RQ (rel. surgery)	1	2.2 ##	1	1.4	1	3.2 #
TNF-α: IL10	Whole Blood	Mean RQ (rel. surgery)	1	1.4	1	1.1	1	1.4
	PBMC	Mean RQ (rel. surgery)	1	1.0	1	1.3	1	1.5

Mean RQ values are calculated from the mean ΔCT values.

# p < 0.05 ΔCT Surgery Vs Multimodal. ## p < 0.01 ΔCT Surgery Vs Multimodal

RQ = Relative Quantity of mRNA expression. rel = relative to. ΔC_T = average CT target - average C_T endogenous control

6.4.8 Comparison of TNF-α: IL-10 mRNA expression in whole blood and PBMCs following oesophagectomy in surgery and multimodal patients

The Ratio of mRNA expression of pro-inflammatory TNF-α to anti-inflammatory IL-10 was compared between the surgery and multimodal groups at each time-point. This provided a measure of the level of pro-inflammatory cytokine expression compared to anti-inflammatory cytokine expression. The $\Delta\Delta C_T$ values (ΔC_T TNF-α - ΔC_T IL-10) for whole blood and PBMC samples, for the surgery and multimodal groups, pre-op and on pod 1 and pod 7 are shown (table 6.6 and figure 6.5). The relative quantity (RQ) of TNF-α mRNA expression, relative to IL-10, again for whole blood and PBMC samples, for both groups, at the three time points are shown (table 6.7).

Preoperatively, in both groups, whole blood TNF-α mRNA expression was greater than IL-10 mRNA expression (mean RQ TNF-α: IL-10: surgery group 55, multimodal group 86). The ratio of whole blood TNF-α: IL-10 mRNA expression fell significantly in both groups on pod 1 (mean RQ TNF-α: IL-10 mRNA expression: surgery group from 55 to 4.7, p = 0.008 and multimodal group from 86 to 4.9, p = 0.004. On pod 7 the ratio of whole blood TNF-α: IL-10 mRNA expression remained significantly lower than pre-op in the multimodal group; the mean RQ TNF-α: IL-10 was 31 versus 86 (p = 0.018). There were no significant differences in the ratio of whole blood TNF-α: IL-10 mRNA expression between patients in the surgery and multimodal groups, overall using an area under the curve (AUC) analysis (p = 0.244), or at any individual time point.

Results for PBMC TNF-α: IL-10 mRNA expression were similar to the findings for whole blood. Pre-op, the mean relative quantity (RQ) TNF-α: IL-10 expression was 39 for the surgery group and 58 for the multimodal group. This decreased significantly on pod 1 compared to pre-op in the surgery group (mean RQ TNF-α: IL-10: from 39 to 2.7 (p = 0.002). On pod 7 the ratio of PBMC TNF-α: IL-10 mRNA expression remained significantly lower than pre-op in the surgery group; the mean RQ TNF-α: IL-10 fell from 39 to 7 (p = 0.003). For the multimodal group, there were trends towards a fall in the RQ of PBMC TNF-α: IL-10 mRNA expression from pre-op to pod 1 and pod 7; however these did not reach statistical significance. When $\Delta\Delta C_T$ values of PBMC TNF-α: IL-10 mRNA expression were studied in the multimodal group, statistical significance was reached (table 6.6). Again, there were no significant differences in the ratio of PBMC TNF-α: IL-10 mRNA expression between patients in the surgery and multimodal groups, overall using an area under the curve (AUC) analysis (p = 0.664), or at any individual time-point.

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6.4.9 The effect of postoperative complications on cytokine mRNA expression

In designing this study, we did not aim to compare the cytokine responses in patients with or without complications following oesophagectomy; because we were already comparing surgery and multimodal treatment groups, our study was underpowered to examine this. We were unsurprised to find no significant differences in the cytokine responses in patients with or without complications, in the surgery or multimodal groups at an RNA level.

6.5 Discussion

The primary endpoint of chapter 6 was to examine the cytokine immune response following oesophagectomy at an RNA level for two cytokines, IL-10 and TNF-α. This was performed for patients treated with surgery alone and for patients treated with multimodal therapy i.e. combined CRT before surgery. The cytokine response following oesophagectomy has been principally examined at a protein level. Chapter 5 examined this more extensively than previously possible using recently developed array technology. In chapter 6, for the first time, the postoperative cytokine responses at protein and RNA levels are compared.

The second endpoint of chapter 6 was to compare the postoperative cytokine responses following oesophagectomy in patients treated with surgery alone or a multimodal treatment regime. This study compared the cytokine response at an RNA level for 8 of the surgery patients and 10 of the multimodal patients. This is the first study, to our knowledge where perioperative cytokine mRNA expression following oesophagectomy has been compared in oesophageal cancer patients treated with surgery alone or multimodal therapy.

Real time quantitative polymerase chain reaction (PQ PCR) was used to measure the cytokine response at an RNA level. PCR was performed for interleukin 10 (IL-10) and tumour necrosis factor alpha (TNF-α), described as the "prototypic proinflammatory and anti-inflammatory cytokines" (Duggan et al., 2006). Although peripheral blood mononuclear cells (PBMCs) are believed to be the main source of IL-10 and TNF-α, RNA was extracted from whole blood in addition to PBMCs for completeness. Multiple PCR reactions were performed for targets (IL-10 and TNF-α), the endogenous control (ABL) and negative control samples; in total, 1,200 PCR reactions were performed.

When the patient characteristics, operative and pathological parameters of the surgery and multimodal group patients, studied in chapter 6 were compared, there was only one significant difference. This was an increased body mass index (BMI) in multimodal patients compared to surgery patients. However, analysis of IL-10, TNF-α and TNF-α: IL-10 mRNA expression of all patients, comparing those greater or less than the mean BMI and those who were normal weight, overweight and obese revealed no differences. Similar serum cytokine results were found following laparotomy or laparoscopy where postoperative cytokine levels were unaffected by patient age or BMI (Torres et al., 2007).

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The postoperative course was similar with regard to complications and the number of days spent in the high dependency or intensive care unit. The postoperative inpatient stay was longer in the surgery group. The multimodal patients in this study were all admitted to hospital for preoperative CRT; this probably allowed for easier hospital discharge planning following surgery.

We studied the cytokine response following oesophagectomy in surgery and multimodal patients at an RNA level for two cytokines, anti-inflammatory IL-10 and pro-inflammatory TNF-α.

In chapter 5, we found no significant change in anti-inflammatory IL-10 levels following oesophagectomy in either the surgery or multimodal patient groups. Serum IL-10 levels proved to be low with a peak mean value of 4pg/ml in the surgery group, and 2pg/ml in the multimodal group occurring on the pod 1. Conversely, a significant postoperative IL-10 response was found in chapter 6; whole blood and mononuclear cell (PBMC) IL-10 mRNA expression levels increased significantly on pod 1 in both groups of patients. Whole blood and PBMC IL-10 mRNA expression levels fell on pod 7; however in the surgery group they remained higher than pre-op levels. As discussed in chapter 5, significant serum or plasma IL-10 responses were previously found following oesophagectomy (Sato et al., 2002, Sato et al., 2001, Yamaguchi et al., 2006, Yamauchi et al., 1998), abdominal aortic aneurysm repair (Bown et al., 2003) and liver resection (Kimura et al., 2006). Plasma IL-10 levels have been found to correlate with the extent of surgery; increases were found following oesophagectomy and pulmonary lobectomy, but not following mastectomy or laparoscopic cholecystectomy (Yamauchi et al., 1998). Increased plasma IL-10 levels have been associated with increased morbidity; following liver resection they correlated with postoperative infections (Kimura et al., 2006) and in severely injured patients detectable IL-10 was associated with the development of sepsis (Sherry et al., 1996). PBMC IL-10 mRNA expression levels have been examined following cardiac surgery with cardiopulmonary bypass; these were increased at 1 and 6 hours postoperatively. This increase was greater at both time-points in patients who did not require ionotropic support following surgery (more favourable) than those who did (Duggan et al., 2006). Whole blood IL-10 mRNA expression levels following ICU admission with sepsis have been investigated; in contrast to the previous study these were significantly up-regulated at 24 hours in patients who did not survive but not in survivors (Abe et al., 2007).

In chapter 5, we found no significant difference in the postoperative IL-10 response in patients treated with surgery or multimodal therapy. In chapter 6 there were no overall differences in whole blood or PBMC IL-10 mRNA expression levels between the surgery and multimodal patient groups, when examined using an area under the curve (AUC)

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analysis. In addition, there were no intergroup differences in whole blood or PBMC IL-10 mRNA expression at pre-op, pod 1 or pod 7 time points. This is the first study, to our knowledge, where the postoperative IL-10 response to surgery in patients treated with preoperative CRT or surgery alone has been investigated. Stimulated T cell IL-10 secretion in oesophageal cancer patients was previously found to remain unchanged before and 4 weeks following neoadjuvant CRT (Heidecke et al., 2002).

In chapter 5, we did not demonstrate any postoperative change in pro-inflammatory serum TNF-α levels in the surgery or multimodal groups. In chapter 6, in multimodal patients, there was no significant postoperative change in whole blood or PBMC TNF-α mRNA expression levels matching the serum findings. However, in the surgery group, there was significantly increased whole blood and PBMC TNF-α mRNA expression on pod 1. As discussed in chapter 5, plasma TNF-α levels were previously undetectable after oesophagectomy, gastrectomy, mastectomy and laparoscopic cholecystectomy (Yamauchi et al., 1998) and levels remained unchanged following abdominal aortic aneurysm repair (Bown et al., 2003). In patients treated with surgery alone for rectal cancer, serum TNF-α levels increased on pod 5 (Wichmann et al., 2003). In patients treated with preoperative CRT, serum TNF-α levels previously remained unchanged during CRT for oesophageal cancer (Zemanova et al., 2005), and were unchanged after CRT and after surgery for rectal cancer (Wichmann et al., 2003). TNF-α mRNA expression levels have been examined following cardiac surgery with cardiopulmonary bypass; Whole blood expression at 6 hours postoperatively (Zimmermann et al., 2003), and PBMC expression at 1 and 6 hours postoperatively have been found to be significantly reduced (Duggan et al., 2006). The reduction in PBMC TNF-α expression was greater at both time-points in patients who did not require ionotropic support following surgery (more favourable) than those who did (Duggan et al., 2006).

In chapter 5, we found no significant difference in the postoperative TNF-α response in patients treated with surgery or multimodal therapy. In chapter 6, preoperative whole blood and PBMC TNF-α mRNA expression were significantly increased in multimodal compared to surgery patients. Postoperative PBMC TNF-α mRNA expression levels were also significantly increased in multimodal patients on pod 7. However, there were no overall differences in whole blood or PBMC TNF-α mRNA expression levels between the surgery and multimodal patient groups, when examined using an area under the curve (AUC) analysis. Previously, postoperative serum TNF-α levels following rectal cancer surgery were found to be significantly higher on pod 1, pod 2 and pod 5 in patients treated with surgery alone compared to those treated with preoperative CRT (Wichmann et al., 2003).

Considering our IL-10 and TNF-α serum findings together, in chapter 5, we found no difference in the postoperative serum responses in patients treated with surgery alone or multimodal therapy. In both groups, there was a lack of a significant pro-inflammatory TNF-α or anti-inflammatory IL-10 response mounted following oesophagectomy. Numerically however, serum TNF-α levels exceeded IL-10 levels at all time-points in both study groups.

Examining IL-10 and TNF-α mRNA expression in chapter 6, there were several differences in TNF-α expression between the surgery and multimodal groups at individual time-points, but not overall, using an area under the curve (AUC) analysis. Pro-inflammatory TNF-α mRNA expression was enhanced in multimodal group patients compared to surgery group patients pre-operatively (Whole blood and PBMC samples) and on pod 7 (PBMC sample). Anti-inflammatory IL-10 mRNA expression (Whole blood and PBMC samples) was similar in both surgery and multimodal study groups. The ratio of TNF-α: IL-10 mRNA expression provided a measure of the level of pro-inflammatory cytokine expression compared to anti-inflammatory cytokine expression in whole blood and mononuclear cells at each time-point. Enhanced pro-inflammatory TNF-α expression relative to anti-inflammatory IL-10 expression was seen pre-operatively in both patient groups. This ratio fell significantly post operatively on pod 1, remaining significantly lower on pod 7 in surgery and multimodal patients. However pro-inflammatory cytokine expression still outweighed anti-inflammatory cytokine expression on pod 1 and pod 7. Multimodal therapy patients demonstrated significantly increased perioperative levels of pro-inflammatory TNF-α mRNA expression compared to surgery patients (described above). However, there were no significant differences between groups when the ratios of TNF-α: IL-10 mRNA expression were compared at each time-point. In fact, the ratios of TNF-α: IL10 expression were almost identical in surgery and multimodal patients preoperatively and on pod 1 and pod 7. Indeed, across the IL-10, TNF-α and TNF-α: IL-10 PCR responses, no overall statistically significant differences were found between the groups using an area under the curve (AUC) analysis.

Therefore, we may conclude that preoperative CRT increased the preoperative levels of pro-inflammatory TNF-α expression in multimodal therapy patients compared to surgery alone patients. However, overall whole blood and PBMC IL-10 and TNF-α expression and the ratio of TNF-α: IL-10 mRNA expression remained the same following oesophagectomy in patients treated with or without neoadjuvant chemoradiotherapy.

In chapters 5 and 6, patterns of IL-10 and TNF-α cytokine expression levels at protein and RNA level were not closely matched; significantly increased mRNA expression on pod 1 in both groups for IL-10 and in the surgery group for TNF-α were not translated into significantly higher serum concentrations. Postoperative serum or plasma cytokine levels have been previously found to be inconsistent with mRNA expression levels in humans

(Huang et al., 2007) and in animal models (Brix-Christensen et al., 2003). Reasons for these discrepancies have been considered before (Duggan et al., 2006): PCR technology can detect lower concentrations than protein technology because of amplification. Also, proteins secreted into plasma may bind strongly to receptors and therefore their detection may be reduced. Of course, increases in mRNA expression are not always translated into increased protein production.

In summary, chapters 5 and 6 demonstrate a complex postoperative interplay between pro-inflammatory and anti-inflammatory cytokines in patients treated with surgery or multimodal therapy for oesophageal cancer. Evidence from chapter 6 demonstrates for the first time, to our knowledge, that preoperative CRT enhances preoperative pro-inflammatory TNF-α mRNA expression in oesophageal cancer patients treated with multimodal therapy relative to those treated with surgery alone; however the overall IL-10 and TNF-α postoperative responses to surgery and the ratio of TNF-α: IL-10 mRNA expression in both treatment strategies is indistinguishable.

Differences in mRNA cytokine responses in oesophageal cancer patients treated with surgery alone or multimodal treatment may in future help explain observed differences in post-operative mortality and overall survival between these patient groups (Fiorica et al., 2004, Malthaner et al., 2004, Urschel and Vasan, 2003). Evolving technology may help uncover differences in oesophageal cancer patients treated with surgery or multimodal approaches. RNA microarray studies of blood or proteomic studies of serum in the postoperative period may help uncover further discrepancies in RNA and protein expression between these treatment groups. The main group of complications following oesophagectomy are respiratory complications; a detailed comparison of RNA and protein expression levels in bronchoalveolar lavage fluid and serum may also uncover inconsistencies between surgery and multimodal patients.

Chapter 7

Overview and discussion of thesis

Overview, discussion and potential future research

This thesis is entitled "local and systemic inflammation in oesophageal disease". The overall aims and objectives of this thesis were to examine the role of inflammation, locally and systemically in two different oesophageal disease processes. Local inflammation was investigated in an animal model of oesophageal reflux. The systemic cytokine inflammatory response was investigated in patients undergoing major surgery for oesophageal cancer.

Part A   Animal studies (Chapters 3 and 4)

In an animal model of surgically induced reflux, we investigated the effects of local inflammation on oesophageal injury, possible tumour induction and strategies which may reduce these.

In chapter 3, we established a rat oesophagojejunostomy reflux model for the first time in Ireland. This involved extensive consideration and optimisation of the choice of animal, preoperative preparation, the type of anaesthesia, the surgical technique, the recovery of animals and postoperative care. We optimised the dose and administration method of a COX-2 inhibitor and an antioxidant to use in this model to potentially reduce oesophageal injury and inflammation. In chapter 4, we investigated the degree of oesophageal inflammation in this oesophagojejunostomy model. We compared animals undergoing surgery only (control animals) and those treated following surgery with either an antioxidant (vitamin C 8mg or 28mg/day), a COX-2 inhibitor (Rofecoxib 1mg/day). We compared the percentage oesophageal ulceration determined by image analysis, animal weight and animal survival between groups. Oesophagitis was present in all animals completing the study and was severe in 27 (43%). There was no evidence of Barrett's oesophagus or adenocarcinoma found in our study. The severity of inflammation and esophageal ulceration were not different between experimental groups. In these animal studies, we successfully established a rat oesophagojejunostomy model. In this model of reflux injury, antioxidants and COX-2 inhibitors failed to improve the severe inflammation induced.

In conclusion, this model created severe esophageal reflux with extensive inflammation but no Barrett's oesophagus or adenocarcinoma. In addition, the systemic insult and catabolism was considerable. The study failed to demonstrate a reduction in esophageal inflammation and ulceration using COX-2 inhibitors or two doses of the antioxidant vitamin C. It is evident that gastric exclusion should be avoided in animal models of reflux, and we would suggest that future studies with COX-2 inhibitors, antioxidants and perhaps NF-κB inhibitors should be undertaken in animal models that induce less severe inflammation.

Part B   **Human studies (Chapters 5 and 6)**

In a human model of inflammation following major surgery for oesophageal cancer, we examined the systemic inflammatory cytokine response at protein and RNA levels. This response was studied in patients who were treated with surgery alone or a multimodal treatment protocol and differences between these strategies were analysed.

In the human studies, serum and blood were collected from 31 non-randomised, stage-matched oesophageal cancer patients treated with surgery alone or a multimodal treatment. This was collected pre-operatively and on postoperative days 1 and 7. In chapter 5, we undertook a detailed analysis of the serum cytokine and growth factor response following oesophagectomy using novel "biochip" array technology. In chapter 6 we used real time quantitative polymerase chain reaction (RQ PCR) to examine whole blood and peripheral blood monocuclear cell (PBMC), IL-10 and TNF-α mRNA expression following oesophagectomy. In the protein and RNA studies, the cytokine responses were investigated firstly in surgery and multimodal patients. The responses were then compared between the two groups of patients and comparison was made between the results at protein and RNA levels. We found significant changes in the levels of 5 serum cytokines following oesophagectomy in both patient groups: IL-6, IL-8, VEGF, MCP1 and EGF. There were no differences in the serum results between the surgery and multimodal groups. Significantly increased IL-10 mRNA expression was found in surgery and multimodal patients after oesophagectomy. Increased TNF-α mRNA expression was found in the multimodal group only. Increased preoperative TNF-α expression levels were found in multimodal patients compared to surgery patients; however, overall IL-10 and TNF-α expression following surgery and the ratio of TNF-α: IL-10 expression was the same in both groups. Differences in cytokine responses were found in patients following oesophagectomy at protein and RNA levels as described.

Parallel protein and RNA detection in this thesis using recently developed technologies allows a new understanding of the complex cytokine following oesophagectomy. Subtle differences found between surgery and multimodal patients in this study may improve our understanding of differences in complication rates and survival between these groups. In future studies, evolving technology may help uncover further differences in oesophageal cancer patients treated with surgery or multimodal approaches. RNA microarray studies of blood or proteomic studies of serum in the postoperative period may help uncover more discrepancies in RNA and protein expression between these treatment groups. The main group of complications following oesophagectomy are respiratory complications; a detailed comparison of RNA and protein expression levels in bronchoalveolar lavage fluid and serum may also uncover differences between surgery and multimodal patients.

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Appendix 1

Application to Trinity College Dublin to register as a postgraduate Higher Degree student

UNIVERSITY OF DUBLIN
TRINITY COLLEGE
GRADUATE STUDIES OFFICE

Proposed date of registration	Proposed Degree	
Oct 1 ☐	M.D.	X
April 1 ☐	M.Ch.	
	M.A.O.	
	M.Dent Sc.	

APPLICATION FOR REGISTRATION FOR A PROFESSIONAL HIGHER DEGREE IN THE FACULTY OF HEALTH SCIENCES

PERSONAL INFORMATION SUPPLIED ON THIS FORM IS CONFIDENTIAL AND USED ONLY FOR STATISTICAL PURPOSES

PART A. GENERAL INFORMATION (Please use Block Capitals throughout)

Surname (Last name) ..... Murphy ..... Title : MR MRS MISS **DR**

Other names (in full)..... James Oliver ..... FEMALE **MALE**
(Enter names accurately. If you are admitted, your name for College purposes will be registered as it is entered here).

Correspondence address: ..... 5 Riverston Gardens, Navan Road, Dublin 7. .....

.....

.....

Home Tel: ..... 086 327 4394 ..... Work Tel: ..... 086 327 4394 .....

Date of birth (dd/mm/yy) 09/03/77 . Country of birth. Ireland ..... Nationality. Irish .....

Present appointment: Cancer Research Fellow, Dept Surgery, St. James Hospital.

If you are admitted, will your fees be paid by **yourself** ☒ by grant ☐ otherwise ☐
(Tick as appropriate)

If by grant, state name of authority .....

I apply for registration for the above degree and in support of my application submit the particulars entered on the subsequent pages of this form and these enclosures. (Copies of certificates, transcripts, etc.)

Encl.   (1) Detailed Research Proposal
         (2) Curriculum Vitae
         (3) Copy of MB, BCh, BAO Degree
         (4) Copy of undergraduate academic results
         (5) Copy of MRCSI Certificate
         (6) Copy of animal licence

Usual signature:

Date:

Day	Month	Year

PART B. EDUCATION

B.1. PRIMARY DEGREE:. MB, BCh, BAO, UCD, Dublin . From year: 94 To year: 2000
(name of university)   (year of graduation)

Final Examination: **Honors** / Distinction

B2. BRIEF CURRICULUM VITAE

Please find enclosed Curriculam Vitae

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PART C. PROJECT DESCRIPTION (Detailed proposal enclosed)

C1. TITLE
LOCAL AND SYSTEMIC INFLAMMATION IN OESOPHAGEAL DISEASE

C2. OBJECTIVE (state what the work is expected to establish and explain its significance)

In an experimental rodent model of inflammation, oxidative stress and tumourigenesis, the effects of novel approaches including anti-oxidants and COX-2 inhibition will be evaluated.

Major surgery for oesophageal cancer induces a marked systemic immunoinflammatory response. This may be modulated, perhaps exaggerated, by chemotherapy and radiation therapy which is increasingly administered prior to surgery. A comprehensive analysis of the immuno-inflammatory response in the perioperative period has not been performed, and this study aims to measure postoperative changes in serum cytokine levels and their relative gene expression in whole blood and in isolated mononuclear and polymorphonuclear cell populations.

C3. Brief description of background and relevant literature on this project (use letters as below to identify recent key references):

The incidence of oesophageal adenocarcinoma has increased significantly in the western world. The reflux of gastric acid, bile and digestive enzymes into the oesophagus may initiate a sequence of injury from reflux oesophagitis and Barrett's metaplasia through dysplasia and eventually adenocarcinoma. Oxidative damage and inflammation are proposed to play an important role in this progression [A]. Rat surgical reflux models may produce oesophagitis, Barrett's oesophagus and oesophageal adenocarcinoma. Potential approaches to preventing oesophageal cancer include anti-inflammatories and antioxidants which target mechanisms escalating injury in the refluxing oesophagus [B].

A multimodality approach is increasingly utilised in gastrointestinal cancer although for oesophageal cancer, the benefit of neoadjuvant chemoradiotherapy before surgery versus surgery alone remains controversial [C]. Equally controversial is the rate of postoperative mortality and complications after multimodal therapy compared with surgery alone. The release of cytokines has a central role in the inflammatory response to major surgery and after oesophagectomy, there is an increase in serum interleukins 6, 8 and 10 (IL-6, IL-8, IL-10) compared to less invasive procedures [D]. The effect of neoadjuvant chemoradiotherapy on the cytokine immune response to major surgery remains unclear. In the case of advanced rectal cancer, preoperative chemoradiotherapy produced a detrimental effect compared to surgery alone [E], but to date no study has examined this in oesophageal cancer patients.

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E. MW Wichmann, G Meyer, M Adam, W Hochtlen-Vollmar, MK Angele, A Schalhorn, R Wilkowski, C Muller, FW Schildberg: Detrimental immunologic effects of preoperative chemoradiotherapy in advanced rectal cancer. Dis Colon Rectum 2003, 46:875-87.

2

C4. Outline briefly and clearly the design of the experiments (including human/animal research populations) whereby you expect to attain the objectives of the project:

EXPERIMENT 1- OPTIMISATION OF ANIMAL MODEL OF REFLUX

This study has been reviewed by the Bioethics Committee, Trinity College Dublin. An animal licence and relevant certificates have been obtained from the Department of Health and Children, Ireland (copy of animal licence enclosed). Optimisation of this animal model is required because it has not been employed in this institution before. A broad outline of the surgical procedure follows. Female wistar rats housed under standard conditions will receive commercial chow which will be withheld overnight before surgery with access to water available at all times. General Anaesthesia will be produced by intraperitoneal ketamine and xylazine hydrochloride. Subcutaneous carpofen NSAID analgesia will be administered. Oesophagojejunostomy will be performed by anastomosis of the jejunum to the oesophagus. Subcutaneous normal saline will be administered to compensate for fluid loss and the ears will be punched to enable identification. Rats will be recovered overnight with access to a heating pad and water. Rat chow will be reintroduced the morning following surgery. A further dose of carpofen will be administered if any signs of pain are observed. Rats will be carefully examined on a daily basis and ill rats will be humanely killed. Once optimisation is achieved, the experimental phase of the study can commence.

EXPERIMENT 2- INHIBITION OF COX-2 AND OXIDATIVE PATHWAYS: THE EFFECTS ON OESOPHAGEAL INJURY IN A SURGICAL REFLUX MODEL.

Six weeks postoperatively, surviving healthy rats will be randomized into 4 groups. A control group will receive normal chow and drinking water. Two groups will receive vitamin C dissolved in their drinking water at concentrations of 0.5g and 2.0g vitamin C per liter water. The final group will receive Rofecoxib 4mg/kg/day administered as a daily oral dose of syrup. Rats will be Sacrificed 20 – 24 weeks postoperatively depending on welfare considerations. At study completion halothane will be administered, cardiac puncture and exsanguination will be performed. Rats will then be placed in a CO_2 chamber until death is confirmed. The oesophagus will be removed from the larynx to the jejunal anastomosis. The specimen will be fixed in 10% buffered formalin and then embedded in paraffin. Tissue sections will be stained with haematoxylin and eosin for histopathological analysis to determine the severity of inflammation, evidence of Barrett's oesophagus, dysplasia or carcinoma. Taking into account previous studies a 25% reduction in the incidence of moderate to severe inflammation between the control and treatment groups would be considered important to detect. The number of animals required per treatment group to detect this difference is 13 or 52 overall. In previous models, the mortality rate was 30%, therefore a minimum number of 74 animals would need to undergo surgery. Given the need to optimise the procedure and other unforeseen difficulties, the total number of animals needed is likely to be greater than 74.

EXPERIMENT 3- EFFECT OF PREOPERATIVE CHEMORADIOTHERAPY ON SERUM INTERLEUKIN LEVELS IN PATIENTS UNDERGOING OESOPHAGECTOMY:

Written informed consent will be obtained from participating patients, using a consent form which has received ethical approval by the St James's Hospital and federated Dublin voluntary Hospitals joint Research Ethics Committee. Patients will receive a patient information leaflet, also approved by this ethics committee. Patients undergoing oesophagectomy with or without prior chemoradiotherapy will be recruited to this study. Blood will be taken before neoadjuvant therapy, preoperatively and on days 1 and 7 postoperatively. Serum will be obtained and stored at -70°C until analysis. Biochip array technology (Evidence Investigator™ system, developed by Randox Laboratories) will be used to simultaneously measure the concentration of 12 cytokines and growth factors in serum samples, using a sandwich chemilumiscent immunoassay principle. The proteins measured include: IL-6, IL-8, IL-10, Vascular Endothelial Growth Factor and Tumour Necrosis Factor-alpha. Based on previous studies, we aim to include 30 patients in this experiment, 15 in each group. This would involve the processing and analysis of more than 100 individually collected serum samples, yielding 1,200 results with the biochip array technology. The use of 15 patients in each group will allow detection of a 20-25% difference in serum cytokine and growth factor levels between groups.

EXPERIMENT 4- THE EFFECT OF PREOPERATIVE CHEMORADIOTHERAPY ON WHOLE BLOOD INTERLEUKIN GENE EXPRESSION IN PATIENTS UNDERGOING OESOPHAGECTOMY:

Once again, patients undergoing oesophagectomy with or without prior chemoradiotherapy will be recruited to take part in this study. Blood will be taken before neoadjuvant therapy, preoperatively and on days 1 and 7 postoperatively. To obtain RNA from whole blood, red cell lysis will be performed firstly, followed by white cell lysis and RNA stabilisation. Complimentary DNA will be made from RNA. Real-Time Quantitative (RQ) PCR will be performed on samples to investigate the change in the relative gene expression of cytokines over the postoperative period. Initially triplicate reactions will be performed initially for pro-inflammatory TNF-alpha and anti-inflammatory IL-10 using duplicate endogenous controls. The detection of changes in gene expression between samples is more sensitive and of greater magnitude than the detection of changes in protein levels. The number of patients required to show a difference between groups is therefore less and we aim initially to study 8 patients in each group in experiments 4 and 5. This would allow us to detect a difference in gene expression of about 30% between groups.

EXPERIMENT 5- THE EFFECT OF PREOPERATIVE CHEMORADIOTHERAPY ON MONONUCLEAR AND POLYMORPHONUCLEAR CELL INTERLEUKIN GENE EXPRESSION IN PATIENTS UNDERGOING OESOPHAGECTOMY:

The same patients in experiment 4 will take part in this study and once again, blood will be taken before neoadjuvant therapy, preoperatively and on days 1 and 7 postoperatively. A density gradient solution containing sodium diatrizoate and dextran 500 (Polymorphprep™) will be used to selectively isolate mononuclear and polymorphonuclear cells from anticoagulated blood. RNA will be extracted from both cell populations and RQ PCR will be performed as described, initially for TNF-alpha and IL-10. In total between experiments 4 and 5, in excess of 2,100 PCR reactions will be performed.

The attention of both candidates and heads of department is drawn to the fact that the work presented must be in greater part the original work of the candidate. Where the work of others forms any part of the thesis it must be fully and explicitly acknowledged, whether published or not.

3

C.5 State your previous experience of the methods to be employed:
I have completed my Basic Surgical Training and recieved my MRCSI (copy enclosed). In addition, Narayanasamy Ravi and the staff in the Bioresources unit have been of great assistance in optimising the animal reflux model. Nicola Miller has been invaluable in all aspects of RNA extraction and RQPCR. I have received training to use the Evidence Investigator system for analysing serum samples by Randox Laboratories.

C.6 Publications (list the more recent and relevant and/or other activities associated with your research work:

ORAL PRESENTATION TO DATE:

Inhibition of COX-2 and oxidative pathways as a means to decrease oesophageal injury in a surgical reflux model
JO Murphy, Ravi N, Byrne PJ, McDonald GSA, Reynolds JV
Sylvester O'Halloran Surgical Scientific Meeting, March 2005

C.7 Location of the project and facilities available for this work:
Animal studies: Bioresources Unit (including Luce Hall operating theatre), Trinity College Dublin.

Human studies: Patients will be recruited in St James's Hospital, Dublin 8. Laboratory facilities are located in the Department of Surgery and the Institute of Molecular Medicine, Trinity Centre, St. James's Hospital, Dublin 8.

C.8 Name of supervisor – if your research has been/will be supervised:
Professor JV Reynolds
Department Of Surgery
Trinity Centre
St. James's Hospital
Dublin 8

E-mail   reynoldsjv@stjames.ie
Telephone   00 353 1 608 2189
Fax   00 353 1 454 6534

C. 9 Names of any collaborators in the project:

Animal Studies:
Department of Surgery, St. James's Hospital: Narayanasamy Ravi, Patrick J Byrne, John V Reynolds.
Department of Histopathology, St. James's Hospital: George SA McDonald.

Human Studies:
Department of Surgery, St. James's Hospital: Nicola Miller, Aoife Ryan, Suzanne Rowley, Narayanasamy Ravi, John V Reynolds.

4

PART D. SUPPORTING STATEMENTS

D1.   STATEMENT BY THE CANDIDATE
I have read the document "Doctor in Medicine (M.D.) – Guidelines for Candidates",
and the proposal contained in this application for registration, has been framed in
the context of the guidelines set out in that document.

Signature…………………………………………………… Date………………

D2.   STATEMENT BY THE HEAD OF DEPARTMENT (In which the candidate
has carried out, or is proposing to carry out the research.)

I have examined this application form being submitted by

……………………………………………………………………………………

for registration for the degree of

……………………………………………………………………………

and to the extent that my Department is involved, I certify the accuracy of the
information contained in the application form.

Signature…………………………………………………… Date………………

D3. ADDITIONAL COMMENTS (IF ANY) BY THE HEAD OF DEPARTMENT

D.4 STATEMENT BY ADVISOR OR SUPERVISOR – IF APPROPRIATE

I have examined this application form being submitted by

……………………………………………………………………………………

for registration for the degee of

……………………………………………………………………………………

and I certify that to the best of my knowledge the information contained in the
application is correct.

Signature…………………………………………………… Date………………

5

Appendix 2

Funding for the animal reflux model was provided by the Royal College of Surgeons in Ireland, Research Fellowship in Surgery 2003 and a salary was provided by Trinity College Dublin

Royal College of Surgeons in Ireland

Registrar / Chief Executive
Professor Kevin O'Malley
MD, PhD, DSc, FRCPE, FRCP Glas, FRCPI.

Telephone: 353 1 402 2187
Facsimile: 353 1 810 2901
E-mail: gconroy@rcsi.ie
Website: www.rcsi.ie

kom/gc

Dr James Oliver Murphy
Tierney Street
Ardee
Co Louth

10 April 2003

Dear Dr Murphy

Re: Research Fellowship in Surgery 2003

I am pleased to inform you that you have been awarded a Research Fellowship in Surgery for 2003.   The purpose of this award is to spend one year studying chemoprevention of oesophageal adenocarcinoma by vitamin C in a rat model. As agreed, you will be hosted by the Department of Surgery, Trinity College, Dublin, at St James's Hospital, and I would be grateful if you could liaise with Professor John Reynolds in this regard.
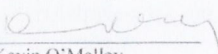
Under the terms of the award, your salary as of 1st July 2003 – at the appropriate stage - will be paid for one year through Trinity College together with €6,350 to cover your research expenses. Please contact Professor John Reynolds regarding the financial details of the Fellowship.

One of the conditions of granting the Fellowship is that you prepare a report on completion of the Fellowship for submission to the College. You may be asked to deliver this report at one of the College's meetings and it may be published in the College Journal. Also, we would ask that the support of the College and Trinity College be acknowledged in any publications arising as a result of this Fellowship and that you will supply us with copies of all such publications at the time they are submitted for publication.

It is planned that a formal presentation of this Fellowship will be made at the RCSI Postgraduate Conferring ceremony in July 2003 and we will be in contact with you regarding this in due course.

May I take this opportunity to congratulate you on being awarded this Fellowship and wish you an enjoyable and rewarding year.

Yours sincerely

Kevin O'Malley
Chief Executive/Registrar

cc: Ms M Alexander   Ms L Loughran   Professor John Reynolds

123, St. Stephen's Green, Dublin 2, Ireland.

Dean: Professor Diarmuid Shanley
Faculty Secretary: Mr Donal Motherway

Trinity College, Dublin 2, Ireland
Telephone: 353-1-608 1476
Fax: 353-1-671 3956
Email:dshanley@tcd.ie
dmotherway@tcd.ie

18 June 2003

Dr James Murphy
Department of Surgery
Trinity Centre
St. James

Dear Dr Murphy

I should have written earlier to congratulate you on your appointment by the University Council to the post of Research Fellow associated with the Department of Surgery, which commenced on 01/07/2003 and is due to terminate on 30/06/2004. We are now catching up on some outstanding issues in Faculty administration.

Your appointment is linked to the continued holding of your hospital/clinical appointment. This letter is being sent to your work address, please advise us if you wish us to use a different address for future communications.

The Faculty is fortunate to have you participate in our teaching and research programmes. We are indebted to you on behalf of students and staff who will benefit from your expertise.

I would be happy to meet you to discuss any issues you might have.

Yours sincerely

Professor Diarmuid Shanley
Dean of Health Sciences

Schools of the Faculty: Physic (Medicine), Dental Science, Physiotherapy, Occupational Therapy, Clinical Speech and Language Studies, Therapeutic Radiography, Nursing and Midwifery Studies

Appendix 3

Animal licence to perform rat oesophagojejunostomy model

B100/3496

08 April 2004

Mr James Oliver Murphy
Department of Surgery
Trinity Centre
St James' Hospital
Dublin 8

**DEPARTMENT
OF HEALTH AND
CHILDREN**
AN ROINN
SLÁINTE AGUS LEANAÍ

Quality and Fairness
A Health System for You

CRUELTY TO ANIMALS ACT, 1876

**As amended by European Communities (Amendment of Cruelty to Animals Act 1876)
Regulations 2002**

A Chara

I am to forward herewith a licence which has been granted to you by the Minister for
Health and Children to enable you to perform experiments on live animals under the
Cruelty to Animals Act, 1876. Certificates A and B have been noted.

Your licence expires on 30th March 2006 if you require any further licences it will be
necessary for you to re-apply before that time. Please allow sufficient time for the
application to be processed. Any new experiment which has not been detailed in your
application for this licence will require re-application.

Mise le meas

**Eilis Gilvarry
Environmental Health Unit**

CRUELTY TO ANIMALS ACT, 1876

As amended by European Communities (Amendment of Cruelty to Animals Act 1876)
Regulations 2002

In exercise of the powers conferred on the Minister for Health and Children by section 8 of the Cruelty
to Animals Act, 1876 a licence is hereby granted to:-
Mr James Oliver Murphy, Department of Surgery, Trinity Centre, St. James' Hospital, Dublin 8.

Subject to the conditions set out on the reverse, for the performance on living animals of the
experiments scheduled beneath, at the following premises:-
Bio Resources Unit, Trinity College Dublin, Dublin 2.

General description and objective of the experiments:-
The study aims firstly to establish and validate an animal model of Barrett's oesophagus using
rats. The second aim is to investigate if vitamin C reduces the incidence of oesophageal
adenocarcinoma and causes a reaction in NF-kB levels in Barrett's oesophagus in a rat model.
Rats that are 4 weeks post oesophagojejunostomy (a model for Barrett's oesophagus and
adenocarcinoma) will be randomised to receive vitamin C (low or high dose), a selective COX-2
inhibitor, a NF-kB inhibitor or a placebo orally in their rat formula/drinking water for
approximately 30 weeks. At this sacrifice will be performed to compare the rate of Barrett's
metaplasia and cancer, and NF-kB activity as well as associated cytokines will be compared in
oesophageal tissue in both study groups. Control rats will undergo no intervention to produce
normal tissue.

Particulars of:-
(a) Type of Animal(s)   Number of each type

   Rat   370 in total

(b) Individual animal use
    Type of animals(s)   Procedure   Frequency and duration of
                                                                 procedure per animal

   Rat   Oesophagojejunostomy   Duration approx. 34 weeks.
                           2-4 weeks after surgery rats Surgery will be performed
                           will receive a once daily oral once per animal. Feeding of
                           dose of low dose vitamin C,   drug or placebo will be
                           high dose vitamin C, a   performed daily. Blood testing
                           selective COX-2 inhibitor, NF- will be performed
                           kB inhibitor or placebo. The 10 approximately monthly per
                           control rats will not receive animal.
                           placebo or drug. Blood testing
                           to monitor serum vitamin/drug
                           levels will be performed.
                           Parenteral administration of
                           drugs may also be used.
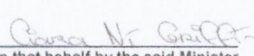   At approximately 28-30 weeks
                           postoperatively rats will be
                           sacrificed.

(c) Type of anaesthetic (if any):- Ketamine, Xylazine and volatile anaesthesia e.g. sevoflurane.

(d) Unless earlier revoked this licence shall remain in force until the 30th day of March 2006.

Signed on behalf of the Minister for Health and Children

Dated this 30 day of March 2004

_____
A person authorised in that behalf by the said Minister

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CONDITIONS

(a)   The experiments shall be performed *only* at the registered premises detailed overleaf and no other premises.

(b)   A written record shall be kept of *every experiment* performed under this licence and such record shall be produced whenever required by the Minister or by an Inspector appointed under the said Act.

(c)   A return shall be furnished to the Minister at such time or times as the Minister may require, in such form and containing such particulars as the Minister may direct, of *all experiments* performed under the said Act by the licensee.

(d)   No experiments using *curare, or agents having similar effects*, may be performed without the special permission of the Minister.

(e)   Only animals from breeding and supplying establishments *registered by the Department of Health* may be used in animal experiments.

(f)   *Every person* performing procedures on an animal must hold a valid licence.

(g)   All animals shall be provided with *housing, an environment, some freedom of movement, food, water and care* which are appropriate to their health and well being.

(h)   The environmental conditions in which animals are bred or kept *shall be checked daily.*

(i)   The animals shall at all times be under the care and supervision of *a competent person.*

(j)   *A named competent person* responsible for the establishment shall be entrusted with the task of administering or arranging for the administration of appropriate care to the animals bred or kept in the establishment.

(k)   Adequate arrangements shall be made for the *provision of veterinary advice and treatment.*

(l)   Any restriction on the extent to which an animal can satisfy its physiological and ethological needs *shall be limited to the absolute minimum.*

(m)   The well being and state of health of the animals shall be observed by a competent person to *prevent pain or avoidable suffering, distress, or lasting pain.*

(n)   The registered place(s) shall be maintained substantially as at the time registration was granted by the Minister.

(o)   The registered place(s) shall be appropriately staffed at all times so as *to ensure the well being of the animals.*

(p)   Practical arrangements must be made to provide for *the care and welfare of animals* at all times, including the holiday periods.

(q)   In any procedure, *the degree of severity imposed shall be the minimum* consistent with the attainment of the objectives of the procedure.

(r)   The licence holder shall ensure that detailed records are maintained of *the source, use and final disposal* of all animals accommodated in the establishment for Scientific purpose and that these records are available for inspection by the Minister for Health or an Inspector appointed by the Minister.

Appendix 4

National animal licence training course certificate, course provided by LAST Ireland (laboratory animal science and training)

National Licencee
Training
Course

30th September & 1st October 2003
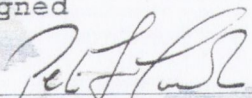
Trinity College Dublin

This is to certify that

LAST-Ireland

Seamus Murphy

Passed an assessment of the course based on the FELASA
recommendations for Category B & C
which implement the European Union Directive 86/609 Article 14.

(Course Content on the back of certificate total lecture time 12.25
hours)

Signed

Peter F. Newlan
Course organiser

Course Content

- An introduction to the history and ethics of animal experimentation (1 hour)

- Animal health - its impact on research (1 hour)

- The use of animals in scientific research - legal controls and applying for a licence (1hour)

- Animal handling / Day-to-day animal care / Procedures video (2 hours)

- Characteristics of laboratory animals; research implications. (1 hour)

- Experimental design / paper critique (1 hour)

- Working safely with animals (0.5 hour)

- Anaesthesia, analgesia and post-operative care (2hours)

- Euthanasia video (0.5 hour)

- Laboratory animals – working in Europe (0.5 hours)

- Alternatives to animals; 'The Options' (1.5 hour)

Contact information:
Bio Resources Unit, Biochemistry Building, Trinity College Dublin, Dublin 2
Phone number: (01) 608 1621
E-Mail: pnowlan@tcd.ie

Appendix 5

E-mail correspondence during the establishment of the rat surgical model of reflux with:

Dr. Ken-ichi Mukaisho and Prof. Takanori Hattori, Department of Pathology, Shiga University of Medical Science, Japan

Appendix 5.1   Initial request for information from Prof. Hattori, sent by e-mail on 25/04/2003.

James Oliver Murphy

From:	James Oliver Murphy	**Sent:**	Fri 25/04/2003 17:22
To:	'hattori@belle.shiga-med.ac.jp'		
Cc:	'hqanimal@belle.shiga-med.ac.jp'		
Subject:	Animal model Barrett's Oesophagus		
Attachments:			

FROM

James Oliver (Seamus) Murphy, MB BCh BAO

100 Birchwood Drive
Springfield
Tallaght
Dublin 24, Ireland

Department of Surgery
Trinity Centre
St James's Hospital
Dublin 8, Ireland

DEAR DR HATTORI

I am a surgical trainee (finishing Basic Surgical Training) and will
commence work in the research laboratory in the Department of Surgery,
St James's Hospital and the Bioresources Unit, Trinity College Dublin,
Ireland, under Professor JV Reynolds.

We hope to establish a rat model of Esophageal Adenocarcinoma and
Barrett's Esophagus. We are extremely interrested in the model of gastro-duodeno-oesophageal reflux used in your
1996 research "Reflux of duodenal or gastro-duodenal contents induces esophageal carcinoma in rats" (Miwa K,
Sahara H, Segawa M, Kinami S, Sato T, Miyazaki I, Hattori T.Int J Cancer. 1996 Jul 17;67(2):269-74).

We would be grateful if you could provide us with more details of the
animal care protocol used in your model. We are especially interrested
in the immediate post-operative period e.g. How were the rats caged
during the first 24 hours, was bedding provided or were wire bottom
cages used to prevent eating of bedding.

Many thanks for your attention and I look forward to your reply

Yours Sincerely

Seamus Murphy

Appendix 5.2 Reply received from Dr. Ken-ichi Mukaisho, sent by e-mail on 03/05/2003.

James Oliver Murphy

From:	向所　賢一 [mukaisho@belle.shiga-med.ac.jp]	**Sent:** Sat 03/05/2003 05:41
To:	James Oliver Murphy	
Cc:		
Subject:	Answer for your question in spite of Prof. Hattori	
Attachments:		

Dear DR James Oliver Murphy

I am an assistant of Department of pathology,　Shiga Univ. of Med. Science.
I will answer your question for animal model in spite of Prof. Hattori.
You had better take care rats till sacrifice, using wire bottom cages used
to prevent eating of bedding. Because, if rats eat tip of wood (bedding),
rats will be died for stenosis of esophagus. The tip of wood is so hard that
it can not go through the anastomosis. We always use the wire bottom cages
used to prevent eating of bedding for long time (till sacrifice). And
animals were allowed to drink water 12 hours after the operation and to eat
36 hours later.
We hope your success of establishing a rat model of Esophageal
Adenocarcinoma.

Yours sincerely,

Ken-ichi Mukaisho

Mailing address: K.MUKAISHO
Tel.: +81-77-548-2168, Fax.: +81-77-543-9880
E-mail: mukaisho@belle.shiga-med.ac.jp
Department of Pathology, Shiga University of Medical Science,
Seta-tsukinowa-cho, Ohtsu, Shiga, 520-2192, Japan

https://webmail.rcsi.ie/exchange/JMurphy5/Inbox/Answer%20for%20your%20questi...   27/01/2008

Appendix 5.3 Request for further information following initial difficulty, sent by e-mail on 13/08/2003.

James Oliver Murphy

From:	James Oliver Murphy	**Sent:** Wed 13/08/2003 15:48
To:	'mukaisho@belle.shiga-med.ac.jp'	
Cc:		
Subject:	Request for Information on Rat Reflux Model	
Attachments:		

From:

James O. Murphy, MB BCh BAO, AFRCSI,
Dept of Surgery, Trinity Centre, St. James's Hospital, Dublin 8, Ireland
Email jmurphy5@rcsi.ie, Phone +353 86 327 4394

Narayanasamy Ravi, MB BS, MS, FRCSI,
Lecturer in Surgery, Trinity Centre, St. James's Hospital, Dublin 8
Email ravin@tcd.ie, Phone +353 86 602 5276

August 13, 2003

Dear Dr Ken-ichi Mukaisho

I am a surgical trainee and have commenced a research fellowship with Professor JV Reynolds in the Department of Surgery, St. James's Hospital and Trinity College Dublin, Ireland. We have a busy upper gastrointestinal unit and are attempting to establish an animal model of Esophageal Adenocarcinoma in Barrett's Esophagus.

I was in contact with you in May this year and you kindly replied with some good advice about post-operative management of rats in wire bottom cages without bedding.

Firstly our congratulations to you on your publication in July's edition of The Scandinavian Journal of Gastroenterology: "Cell Kinetic Study on Histogenesis of Barrett's Esophagus Using Rat Reflux Model". Your Model is very elegant, maintaining stomach function unlike the esophago-jejunostomy model and avoiding deforming the stomach as in Chen's model.

The findings that the columnar epithelium in Barrett's is metaplastic and that proliferative cells have probably bidirectional differentiation potential are of great importance. So too is the concept that GRCL is possibly related to carcinogenesis associated with chronic inflammation throughout the gastrointestinal tract.

Regarding our own attempts to set-up an animal model of Esophageal cancer, unfortunately we have encountered a lot of difficulty. We have performed esophago-jejunostomy on 20 female Wistar rats and have had a high mortality rate. Autopsy revealed anastomotic leakage or breakdown in 12 (60%) cases.

We have kept detailed operative and postoperative records and have taken numerous digital images and would be happy to forward these to you for review.

Your new model has been used with great success and you have demonstrated a high tumour yield at 50 weeks and a very low mortality rate. While we are attempting a different, more traditional model we feel you would be ideal to advise us on our anastomotic technique and post-operative care.

We would appreciate greatly if you could provide us with a detailed description of your anastomotic procedure and also your detailed postoperative care protocol.

Many Thanks
Yours sincerely

James Murphy

https://webmail.rcsi.ie/exchange/JMurphy5/Sent%20Items/Request%20for%20Inform...   27/01/2008

Appendix 5.4 Reply received from Prof. Takanori Hattori, sent by e-mail on 14/08/2003.

James Oliver Murphy

From:	Hattori [hattori@belle.shiga-med.ac.jp]	Sent: Thu 14/08/2003 04:50
To:	James Oliver Murphy	
Cc:		
Subject:	Barrett Operation	
Attachments:		

Dear Dr. James O. Murphy

I am a boss of Dr. Mukaisho, and thank you for your comment on our recent work.

The reflux operaton using rats is not easy. In our previous studies with Prof. Miwa, mortality had been as high as 50% in the reflux operation of the stomach. But, our method recently reported in Scand. J. Gastroenterol. is much easier. The mortality is less than 10% now. Please take care of anastomosis, avoiding the damage of large arteries of the esophagus. After several failures, you will find out a clue to overcome the difficulty.

The easiest way is to come here and to study how to operate. We are always ready to accept you.

Our GRCL is a modified UACL theory of Nick Wright, a intimate friend of mine, and we are doing several cooperative works. The UACL is good theory, but Wright and colleagues had not observed the genesis of gastric (foveolar) cell types and of intestinal goblet cells. Another work of GRCL will appear soon in Digestive Disease and Science.

\##

服部隆則	Takanori HATTORI, MD, Ph.D.
滋賀医科大学第一病理	Dept Pathol Shiga Univ Med Sci
大津市瀬田月輪町	Seta Ohtsu, Japan 520-2159
TEL 077-548-2166	TEL +81-77-548-2166
FAX 077-543-9880	FAX +81-77-543-9880

\##

https://webmail.rcsi.ie/exchange/JMurphy5/Inbox/Barrett%20Operation.EML?Cmd=...   27/01/2008

Appendix 5.4 Reply to Prof. Takanori Hattori following success in establishing model, sent by e-mail on 25/08/2003.

James Oliver Murphy

From:	James Oliver Murphy	Sent:Mon 25/08/2003 14:30
To:	'Hattori '	
Cc:		

Subject: RE: Barrett Operation
Attachments:

Dear Professor Hattori

Thank you for your reply to our query and your advice. Happily we can report some good news since we contacted you last. We have changed our technique of anastomosis and have performed it on several rats without mortality in the first week post op.

We are using intraperitoneal ketamine and xylazine for anaesthesia- This was previously used for rat surgery at our institution and is also the method used by Chen and Yang in their model.

We are now also giving subcutaneous Co-Amoxyclav (Augmentin) pre op.

Our anastomosis is an end to side oesophago-jejunostomy, made about 3cm from ligament of Trietz. We are performing suture ligation at the gastro-oesophageal junction and deviding the oesophagus 2mm above this.

In Total we are placing 8 x 7/0 full thickness polypropylene sutures. We place 2 angle stay sutures, followed by 3 anterior wall sutures. We then turn the anastomosis to place 3 posterior wall sutures.

We are closing abdominal wall and then skin with continuous 5/0 braided absorbable material (Lactomer/Polysorb). We then give the rats 5 mls subcutaneous saline and NSAID (1.25mg carpofen) analgesia.

Post Op we are placing rats in single wire bottom cages. For the first night we give them oral glucose solution. On the first day we are giving natural yoghurt and gradually we introduce rat pellet diet. For pain relief we are using once daily NSAID (carpofen) for the first 3-4 days. During the first 2 weeks of recovery we have also given chocolate and peanuts while progressing to full pellet diet.

Once again thank you for your reply. Many Thanks also for your kind offer to visit your institution and study the procedure. Unfortunately we do not have sufficient funding to allow this at present, but we will keep your offer in mind and hope to visit you in the future.

I will contact you again to let you know how we are progressing with our model. If you have any comments on our technique or post op care we would be very grateful.

Thank you
Yours sincerely, James Murphy.

https://webmail.rcsi.ie/exchange/JMurphy5/Sent%20Items/RE:%20Barrett%20Operat... 27/01/2008

Appendix 6

E-mail correspondence to establish which COX-2 inhibitor to administer in the rat surgical model of reflux with:

Vivianne Khoury, Dr. Ian Rodger, Dr. Pang

and Dr. Robert Young,

Merck Frosst Canada & Co. Quebec, Canada

and Merck & Co., New Jersey, USA

Appendix 6.1   Initial inquiry about the availability of MF-Tricyclic, sent by e-mail on 09/06/2003.

James Oliver Murphy

From:	James Oliver Murphy	Sent: Mon 09/06/2003 16:58
To:	'viviane_khoury@merck.com '	
Cc:		
Subject:	MF-Tricyclic	
Attachments:		

FROM

James Oliver (Seamus) Murphy, MB BCh BAO, AFRCSI

Tierney Street
Ardee
Co Louth
Ireland

Department of Surgery
Trinity Centre
St James's Hospital
Dublin 8, Ireland

Dear Ms Khoury

I am a surgical trainee and will commence work in the research laboratory in the Department of Surgery, St James's Hospital and the Bioresources Unit, Trinity College Dublin, Ireland, under Professor JV Reynolds.

We hope to establish a rat model of Esophageal Adenocarcinoma and
Barrett's Esophagus. Part of our research will study the chemoprevention of adenocarcinoma using selective COX-2 inhibitors.

We hope to use MF-Tricyclic for this purpose. This is similar to a study performed last year and the authors stated in their paper that the MF-Tricyclic was provided by Merck Frost, Montreal, Canada:

(Buttar NS, Wang KK, Leontovich O, Westcott JY, Pacifico RJ, Anderson MA, Krishnadath KK, Lutzke LS, Burgart LJ. Chemoprevention of esophageal adenocarcinoma by COX-2 inhibitors in an animal model of Barrett's esophagus. Gastroenterology. 2002 April 122(4):1101-1112.)

We would like to approx purchase 40grammes of MF-Tricyclic. We would provide 35 rats with a dose of 10mg/kg/day for approximately 28 weeks.

I have some queries about this product if you are able to find it.

1   Is this drug available in Ireland/UK or could shipping be arranged from canada, and how long would this take? What is the price?

2   What form does the drug come and how long is its shelf-life. We intend to administer it by mixing it with rat chow.

3   We hope to perform blood assays for drug concentration. In the above study this was performed by Merck Frost Canada. Could this be arranged either in the UK or else in Canada.

Many Thanks

Yours Sincerely

Seamus Murphy

https://webmail.rcsi.ie/exchange/JMurphy5/Sent%20Items/MF-Tricyclic.EML?Cmd=... 27/01/2008

Appendix 6.2   Initial reply about the availability of MF-Tricyclic, sent by e-mail on 09/06/2003.

James Oliver Murphy

From:	Khoury, Viviane [viviane_khoury@merck.com]	**Sent:** Mon 09/06/2003 20:56
To:	James Oliver Murphy	
Cc:		
Subject:	FW: MF-Tricyclic	
Attachments:		

Dear Dr. Murphy,

Please see below the response I got from our 2 scientists.

Good luck in your search!

Viviane Khoury
Office of Dr. Sam McClintock
Pharmaceutical Research & Development
Merck Frosst Canada & Co.
www.merckfrosstlab.ca
Tel: (514) 428-8634
Fax: (514) 428-8677
viviane_khoury@merck.com

-----Original Message-----
From: Rodger, Ian W.
Sent: Monday, June 09, 2003 3:42 PM
To: Khoury, Viviane
Subject: RE: MF-Tricyclic

Hi Viviane,
We do not have sufficient MF-tricyclic left to accommodate this researcher's request.
regards

Ian
Ian W. Rodger,
Executive Medical Director,
Mail Drop WS3D-18
Tel#: (908) 423-4110
Fax#:(908) 423-1797
E-mail: ian_rodger@merck.com

Appendix 6.3   Second inquiry about the availability of MF-Tricyclic, sent by e-mail on 10/06/2003.

James Oliver Murphy

From:	James Oliver Murphy	Sent: Tue 10/06/2003 14:27
To:	'ian_rodger@merck.com '	
Cc:		
Subject:	MF-Tricyclic	
Attachments:		

Dear Dr Rodger

Many Thanks for your prompt reply to my query.

Do you know if MF-Tricyclic is made/available anywhere else or was it made exclusively by Merck·Frosst Canada?

Secondly If you have a small amount of it available, I would be interrested because we could make our sample size smaller. In our experiment the MF-Tricyclic group will be acting as a control.

Thank You

James Murphy

Appendix 6.4   Reply from Dr. Ian Rodger about the availability of MF-Tricyclic, sent by e-mail on 10/06/2003.

James Oliver Murphy

From: Rodger, Ian W. [ian_rodger@merck.com]   Sent: Tue 10/06/2003 17:56
To: James Oliver Murphy
Cc:
Subject: RE: MF-Tricyclic
Attachments:

James,
MF-tricyclic was one of a series of Cox-2 inhibitors that we made as part of
the Vioxx discovery programme. There are only about 2mg of substance left
so that there is no way that it would be adequate for your needs. We will
not be making any more of the material so I cannot see any way forward for
you with this compound.
regards

Ian
Ian W. Rodger,
Executive Medical Director,
Mail Drop WS3D-18
Tel#: (908) 423-4110
Fax#:(908) 423-1797
E-mail: ian_rodger@merck.com

Appendix 6.5 Inquiry about the availability of rofecoxib, its addition to standard chow and monitoring of rofecoxib blood levels, sent by e-mail on 26/06/2003.

James Oliver Murphy

From:	James Oliver Murphy	Sent: Thu 26/06/2003 07:28
To:	'hok_pang@merck.com '	
Cc:		
Subject:	Vioxx for research	
Attachments:		

FROM

James Oliver (Seamus) Murphy, MB BCh BAO, AFRCSI

Department of Surgery
Trinity Centre
St James's Hospital
Dublin 8, Ireland

Dear Dr. Pang

I spoke with the Vioxx representative in our hospital, Niamh Mc Evoy and she recommended I direct my query at yourself.

I am a surgical trainee and I commence work in the research laboratory in the Department of Surgery, St James's Hospital and the Bioresources Unit, Trinity College Dublin, Ireland, under Professor JV Reynolds.

We are establishing a surgical rat model of Esophageal Adenocarcinoma and Barrett's Esophagus. Our research will study the chemoprevention of adenocarcinoma using selective COX-2 inhibitors and other compounds.

We had orignally hoped to use MF-Tricyclic for this purpose, similar to a study performed last year:

(Buttar NS, Wang KK, Leontovich O, Westcott JY, Pacifico RJ, Anderson MA, Krishnadath KK, Lutzke LS, Burgart LJ. Chemoprevention of esophageal adenocarcinoma by COX-2 inhibitors in an animal model of Barrett's esophagus. Gastroenterology. 2002 April 122(4):1101-1112.)

MF-Tricyclic in this study was provided by Merck Frost, Montreal, Canada who also provided rat serum MF-Tricyclic levels.

I previously contacted Dr Ian Rodger, Merck Frost Canada, however MF Tricyclic is no longer available:

James,
MF-tricyclic was one of a series of Cox-2 inhibitors that we made as part of the Vioxx discovery programme. There are only about 2mg of substance left so that there is no way that it would be adequate for your needs. We will not be making any more of the material so I cannot see any way forward for you with this compound.

regards

Ian
Ian W. Rodger,
Executive Medical Director,
Mail Drop WS3D-18
Tel#: (908) 423-4110
Fax#:(908) 423-1797

https://webmail.rcsi.ie/exchange/JMurphy5/Sent%20Items/Vioxx%20for%20research...   27/01/2008

Appendix 6.5 (continued)

E-mail: ian_rodger@merck.com

We are therefore considering using another COX 2 inhibitor, e.g. rofecoxib as part of our study.

We have some queries about it-
1   Are comercial Vioxx preparations water soluble (either capsules or suspension). we intend to administer it by mixing it with either drinking water or admixed in rat chow.

2   We hope to perform blood assays for drug concentration to show rats are recieving an adequate dose. In the above study MF-Tricyclic levels were performed by Merck Frost Canada. Could this be arranged through merck for Vioxx?

We commence our research in the next 2-3 weeks and I would be grateful for a prompt response. I would be happy to provide further information if needed.

Many Thanks

Yours Sincerely

Seamus Murphy

https://webmail.rcsi.ie/exchange/JMurphy5/Sent%20Items/Vioxx%20for%20research...   27/01/2008

Appendix 6.6   Reply from Dr. Robert Young about the use of rofecoxib, sent by e-mail on 17/06/2003.

James Oliver Murphy

From:	McCusker, Kathy [kathy_mccusker@merck.com]	**Sent:** Tue 17/06/2003 17:02
To:	James Oliver Murphy	
Cc:	Foster, Joanna	
Subject:	FW: MFCC Web Feedback	
Attachments:		

Dear Dr. Murphy,

Please see message below form Dr. Robert Young, vice-president of Medicinal Chemistry.

Thank-you,

Kathy

Kathy McCusker
Merck Frosst Canada & Co.
16711 TransCanada Highway
Kirkland, Quebec H9H 3L1
CANADA
Tel: (514) 428-3249
Fax: (514) 428-8535
kathy_mccusker@merck.com

-----Original Message-----
From: Young, Robert
Sent: Monday, June 09, 2003 5:18 PM
To: McCusker, Kathy
Subject: RE: MFCC Web Feedback

This could potentially be supported with drug formulated in chow but the proposal would need to be evaluated by our Cancer committee which would review the study design and planned protocol with expected outcomes. If approved the drug in chow would be supplied free of charge and would be shipped from Canada. We will need to have a plan (3 to 5 pages maximum) and it will require 2-3 months to present to the committee and get a decision. We cannot offer drug analysis in blood normally.

Bob

Appendix 7

ST. JAMES'S HOSPITAL

James's Street, Dublin 8

Telephone (+353 1) 410 3000

www.stjames.ie

MEDICAL PERSONNEL DEPARTMENT

2nd December 2005,

To whom it may concern.

This is to confirm that **Dr. Seamus Murphy** is employed by St. James's Hospital as a Registrar in General Surgery from 1st July 2005 to 30th June 2006 inclusive. Previous to this he was employed as Cancer Research fellow from 1st July 2004 to 30th June 2005. He was awarded a Clinical Research Fellow Certificate by St. James Hospital in 2004.

If you have any further queries, please do not hesitate to contact us.

Yours sincerely,

Louise Whelan.
Medical Personnel Department
Tel: 01 416 2255/2257
Fax: 01 410 3465
E-mail: nchdinfo@stjames.ie

University Teaching Hospital of Trinity College Dublin

Appendix 8

Patient information leaflet and consent form. Written consent was obtained from all patients studied in this thesis

ST. JAMES'S HOSPITAL

James's Street, Dublin 8
Information leaflet and Consent Form
Telephone: 353 1 410 3000
For volunteer participation in Research study
www.stjames.ie

JOINT RESEARCH ETHICS COMMITTEE
ST JAMES'S HOSPITAL AND FEDERATED DUBLIN VOLUNTARY HOSPITALS

1. **Title of Research studies:**
   a) **A prospective trial investigating the effects of chemoradiotherapy and surgery on gut barrier integrity and host immune response in oesophageal cancer patients.**
   b) **Relationship of Gene Polymorphisms with Cancer of Oesophagus.**

2. **Introduction:**

We would like to invite you to participate in a research project studying the effects of various aspects of your treatment on the spread of cancer and your ability to fight infection. You are under no obligation to participate and if, when you have read and heard about the study, you would prefer not to do so, we will accept your decision without question.

Some patients who have surgery for cancer develop recurrences that may be due to microscopic deposits of cancer in the bone marrow. We believe that chemotherapy before surgery in certain cases may prevent these from growing. We further believe that chemoradiotherapy and surgery alter the body's response to infection by affecting blood cells as they develop in the bone and in the circulation. We hope to get a better idea of the processes involved in order to improve our management of patients like you.

We are also interested in finding if there is an association between the genes and development of cancer. We are trying to find out how the variability in genes affects the treatment that is given to a patient. In order to understand the development of cancer, we are also studying the non-malignant conditions of reflux oesophagitis and Barretts esophagus. In addition, for comparison we will be examining patients who have no abnormality in the oesophagus. We will analyze the following genes.

- TNF-A and related genes in the Major Histocompatibility Complex region.
- The IL-1 family of genes including IL-1α, IL-1β and their endogenous receptor IL-1ra.
- P-73 and related genes.
- MDR and related drug transporter genes.

3. **Procedure:**

We intend to investigate patients who are having surgery for cancer of the oesophagus, stomach or pancreas throughout their treatment period, including their check examination six months after their operation. It is unlikely that you would be asked to attend extra outpatients' appointments for this study.

Your participation in this study will not affect the way we treat you. All the investigations and tests are standard for someone undergoing surgery with or without chemotherapy apart from the extra blood we will require when you visit us, some tissue samples we shall take during your operation and a bone marrow sample we shall take while you are asleep for your operation. These samples will be used to investigate how your treatment affects your immune system and cancer cells in the bone.

University Teaching Hospital of Trinity College Dublin

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4. Benefits:

This study may have no direct benefit to you but the results may benefit subsequent patients. We will be happy, however, to let you see your results and discuss them with you.

5. Risks:

The investigations that we are performing do not carry any greater risks than the tests you would be having normally during your treatment.

6. Exclusion from participation:

Your doctor has told you that you cannot be in this study if any of the following are true: You have evidence of infection or sepsis, you are receiving total parenteral nutrition, you are taking steroids or non-steroidal anti inflammatory drugs or, you have peptic ulcer disease or inflammatory bowel disease.

7. Alternative treatment:

Your doctor is giving you the most suitable treatment. The use of your blood is not a part of your treatment. You do not have to be a part of this study to be treated.

8. Confidentiality:

Your identity will remain confidential. Your name will not be published and will not be disclosed to anyone outside the hospital. Your blood sample will have a code number assigned to it and the result obtained from your sample will not be traceable to you.

9. Compensation:

No known risks are involved in taking 5 – 10 ml of blood from your body. Your doctors are covered by standard medical malpractice insurance. Nothing in this document restricts or curtails your rights.

10. Voluntary Participation:

You have volunteered to participate in this study. You may quit at any time. If you decide not to participate, or if you quit, you will not be penalised and will not give up any benefits, which you had before entering the study.

11. Stopping the study:

You understand that your doctor may stop your participation in the study at any time without your consent.

12. Permission:

The research study has hospital Research Ethics Committee approval.

13. Further information:

You can get more information or answers to your questions about the study, your participation in the study, and your rights, from _____
who can be telephoned at_____
If your doctor learns of important new information that might affect your desire to remain in the study, he will tell you.

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ST JAMES'S HOSPITAL AND FEDERATED DUBLIN VOLUNTARY HOSPITALS
JOINT RESEARCH ETHICS COMMITTEE

Title of Research studies:

1. **A prospective trial investigating the effects of chemoradiotherapy and surgery on gut barrier integrity and host immune response in oesophageal cancer patients.**
2. **Relationship of Gene Polymorphisms with Cancer of Oesophagus.**

This study and this consent form have been explained to me. My doctor has answered all my questions to my satisfaction. I believe I understand what will happen if I agree to be part of this study.

I have read, or had read to me, this consent form. I have had the opportunity to ask questions and all my questions have been answered to my satisfaction. The doctor has explained to me that my four genes will be typed. These genes are TNF-A, IL-1B, MDR, and P-73. I freely and voluntarily agree to be part of this research study, though without prejudice to my legal and ethical rights. I have received a copy of this agreement and I understand that, if there is a sponsoring company, a signed copy will be sent to that sponsor.

PARTICIPANT'S NAME:
PARTICIPANT'S SIGNATURE:
Date:
(Date on which the participant was first furnished with this form:)

Where the participant is incapable of comprehending the nature, significance and scope of the consent required, the form must be signed by a person competent to give consent to his or her participation in the research study (other than a person who applied to undertake or conduct the study). If the subject is a minor (under 18 years old) the signature of parent or guardian must be obtained:-
NAME OF CONSENTOR, PARENT or GUARDIAN:
SIGNATURE:
RELATION TO PARTICIPANT:

Where the participant is capable of comprehending the nature, significance and scope of the consent required, but **physically unable to sign** written consent, signatures of two witnesses present when consent was given by the participant to a registered medical practitioner treating him or her for the illness.

NAME OF FIRST WITNESS: **SIGNATURE:**
NAME OF SECOND WITNESS: **SIGNATURE:**

Statement of investigator's responsibility: I have explained the nature, purpose, procedures, benefits, risks of, or alternatives to, this research study. I have offered to answer any questions and fully answered such questions. I believe that the participant understands my explanation and has freely given informed consent.
Physician's signature:
Date:
(Keep the original of this form in the participant's medical record, give one copy to the participant, and keep one copy in the investigator's records).

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